THE UNIVERSITY OF ALBERTA

EFFECT OF INORGANIC IONS ON THE RESPONSE OF VASCULAR SMOOTH MUSCLE

A THESIS

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by

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The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Effect of Inorganic Ions on the Response of Vascular Smooth Muscle," submitted by Khem H. Jhamandas in partial fulfilment of the requirements for the degree of Master of Science.

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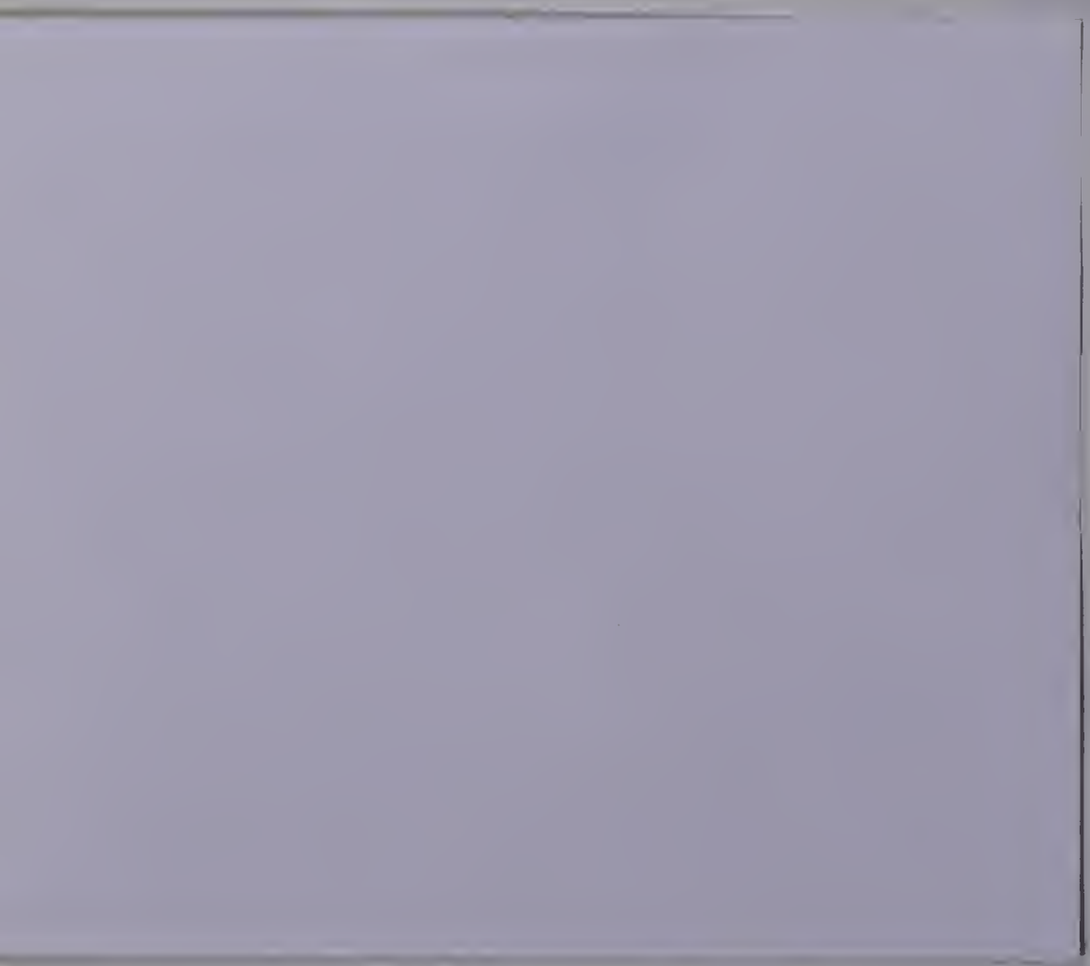

ABSTRACT

To investigate the possibility that the depression of contractile response of vascular smooth muscle to noradrenaline, which is caused by hypercapnia, may be due to an interference with the availability of calcium at some stage in the stimulus-contraction mechanism, the effect of inorganic ions on the contractile response of rabbit aorta tissue was studied. Electrical stimulation of isolated loops of rabbit aorta in Krebs bicarbonate solution causes a dual response consisting of an initial fast contraction and a slow, more prolonged response, the latter being more dependent on the external calcium concentration than the initial response. Both isoproterenol and a high CO_2 concentration in the bath solution reduced the delayed portion of the response but, although dichloroisoproterenol prevented the depressive action of isoproterenol on the delayed response it did not prevent the depression caused by high CO_2. It was concluded that the depression produced by high CO_2 is not mediated through an interaction with β-receptor sites but may be due to a reduction in the availability of calcium at the stimulus-contraction coupling level.

To investigate the possibility that a sodium-calcium competition functions to control alterations in calcium availability for contraction of the vascular smooth muscle, electric current, noradrenaline, and

excess potassium were used as the stimulating agents, and the responses of aorta tissue were plotted against the ratio $[Ca^{++}] / [Na^{+}]^2$ in the external medium. As the responses did not fall on a common regression line but on two separate lines dependent on sodium concentration, it was concluded that no simple sodium-calcium competition for specific anionic site functions to control the response of this muscle.

To investigate the further possibility that high CO_2 may influence the response of vascular smooth muscle through an anionic mechanism, such as an increase in the intracellular bicarbonate ion, the effects of a series of foreign anions on the contractility of the rabbit aorta were examined. The mechanical response varied with the anion present and the stimulating agent used, those induced by potassium being potentiated by bromide, nitrate, iodide and thiocynate anions, while those induced by noradrenaline were only slightly changed or depressed by the anions. The differences in responses induced under the influence of foreign anions were attributed to an alteration in the binding and release of calcium from sites in the membrane and to differences in the sources of calcium utilized to initiate the contraction. It was concluded that high CO_2 like some anions, may produce a depression of contractile response to noradrenaline by influencing the binding, release and re-binding of calcium at its storage sites.

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PUBLICATIONS

Parts of the work described in this thesis have been reported previously as follows:

Nash, C. W. and Jhamandas, K. H., "Depression of Secondary (slow) phase of contraction of rabbit aorta tissue to electrical stimulation by low Ca^{++} and high CO_2," Fed. Proc., 24: 107f (1965).

Nash, C. W., Luschka, E. V. and Jhamandas, K. H., "An Investigation of Sodium and Calcium Competition in Vascular Smooth Muscle," Can. J. Physiol. Pharmacol., 44: 147 (1966).

Nash, C. W. and Jhamandas, K. H., "Reversal of the Effects of Anions on the Response of VSM," Fed. Proc., 25: 351 (1966).

To my parents

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INTRODUCTION

This investigation was carried out to observe the effect of inorganic ions on the contractile response of vascular smooth muscle (VSM) in order to study a possible relationship between the depressive effect of high CO_2 on responses induced by noradrenaline and the availability of calcium for contraction, and to investigate some of the possibilities by which the availability of calcium may be influenced.

The calcium ion, because of its involvement in all stages of the contractile process, is perhaps the most unique of all inorganic ions normally implicated in muscular contraction. A removal of this ion from the external medium and the tissue binding sites, leads to a loss of contractility in all types of muscle. It is possible that the changes in contractility produced as result of exposing the tissues to drugs, ions and other agents, although effected by diverse pathways, may ultimately be related to an increase or a decrease in the availability of calcium ions to the contractile process. It has been suggested by Nash *et al*. (45) that hypercapnic depression may be the result of interference with such calcium availability. However, the mechanism by which high CO_2 may interfere with the role of calcium is not known. It has been proposed that if a sodium–calcium antagonism

exists in the vascular smooth muscle, then a high CO_2 concentration may alter the calcium availability by reducing the formation of an active calcium complex or by promoting the formation of an inactive sodium complex at the site of antagonism and resulting in a depression of contractility. The presence of such an antagonism between sodium and calcium has not so far been shown to function in vascular smooth muscle, although suggestions have been made regarding its existence in this muscle (5, 58). These suggestions rest on the observation that in a low sodium environment, the response of vascular smooth muscle is potentiated.

The evidence from skeletal muscle suggests that foreign anions influence the contractility of this muscle by influencing the binding and the permeation of calcium (3, 21, 51). This is also probable in the case of smooth muscle, in view of the proposed binding sites of calcium (13) and their mobilisation from these sites by stimulating agents such as potassium and noradrenaline (33). Thus an alternate mechanism of the change in calcium availability caused by high CO_2 may be anionic, and this may involve an interference with the binding and release of calcium ions.

The present investigation was carried out to determine whether the depression produced by CO_2 is related to availability of calcium. This included a study of a sodium–calcium antagonism in the vascular smooth muscle, and of the effect of a series of foreign anions on the contractility of vascular smooth muscle.

LITERATURE REVIEW

A. The Effects of Carbon Dioxide and Calcium Availability on the Contractility of Vascular Smooth Muscle.

It has been shown by several workers that hypercapnia causes a decrease in the pressor response to noradrenaline in intact animals (16, 44, 52). In isolated preparations of vascular smooth muscle (VSM) hypercapnia has been shown to exert a depressant effect on the contractility when such preparations are stimulated by noradrenaline. Although several possibilities for the site and the mechanisms of the hypercapnic depression of the response have been suggested it is still not clear which of the stages in the contractile response of VSM are affected by a high CO_2 concentration. Tobian and coworkers (54) using the contractile response of isolated rat aorta showed that the depression of the response may be due to a direct effect on either the receptor mechanism or on the contractile mechanism itself. Sears and Eisenberg (50) on the basis of their work on artificial membrane suggested that CO_2 causes a liberation of hydrated cations from the cell membrane, which tend to decrease the water miscibility of the cell membrane and increase its electrical resistance. These authors suggested that a high CO_2 tension may cause the hypoexcitability of cells

by a decrease in the ease of ion penetration. From the studies on VSM in vitro and in vivo Nash et al. (45) have indicated that the depressive action of high CO_2 on the response of VSM to noradrenaline is exerted at neither the drug receptor reaction nor the final contractile mechanism, and suggested a coupling reaction as the site of the depressant action. Since calcium is an important factor in the coupling of the stimulus to the contractile process, these authors have proposed that a high CO_2 concentration may depress the response to submaximal doses of noradrenaline by influencing the availability of calcium for contraction. The complete depression of response cannot be accounted for in terms of associated low pH as shown by tests using phosphate buffer instead of bicarbonate buffer to alter the pH (45) of the bathing medium. Nash et al. have suggested that if a sodium and calcium competition exists in the VSM, then the CO_2 or the associated low pH may influence the calcium through the alteration in the availability of free sodium ions at the site of competition. Alternatively the depressive action may be due to the high CO_2 concentration changing the availability of the free calcium by affecting the membrane structure. A further possibility is that an associated increase in the bicarbonate ion may account for the hypercapnic depression by influencing the binding, release or rebinding of the membrane calcium.

B. Calcium and Smooth Muscle Contraction.

The contractile response of any muscle is the end product of a fixed series of events: the membrane phenomenon, the coupling process, and the protein contraction. The terminology of these events does not imply that these are discrete and unrelated processes or that they necessarily include all the happenings during a contractile process. The effect of all chemicals on the end product can best be understood by considering them in relation to each of these events. At present time there is considerable evidence that calcium is involved in all three events, although the degree of involvement may vary between events and between muscles.

a. Membrane Phenomena. In general the contractile response seems to be initiated by the action potential although many examples can be cited in which the action potentials occur without a mechanical response or the tension is developed and maintained in the absence of action potentials (1, 19, 26). The latter seem to be the usual triggers for either tonic or phasic contractions but the application of this generalisation to VSM has not yet been established. Although reported membrane and action potentials of VSM (48, 55) are similar to those of other smooth muscle, we are faced by the observations that the contractile response to noradrenaline occurs in the absence of action potentials or change in the resting potentials (2). Waugh (58) using the contractile response of a depolarized VSM has obtained indirect evidence that the contraction occurs in VSM in response to epinephrine and in the absence

of changes in the membrane potential.

According to the ionic hypothesis an action potential, consisting of an initial large inward sodium current followed by an outward potassium current, slightly lowers the ionic gradient across the cell membrane, but a sodium-potassium pump restores the gradients over a period of time. There is a possibility that calcium ions carry the inward current during the rising phase of the action potential. Holman (35) has shown that in taenia coli bathed in calcium free solution the spikes become slower and eventually disappear. Burnstock and Straub (11) have shown that when the smooth muscle of taenia coli is exposed to solutions containing little or no calcium, the membrane potential undergoes an immediate depolarisation of 10 to 20 mV. In the guinea pig taenia coli and progestrone treated uterus this depolarisation appears to be transient, the resting potential recovering towards its normal level in spite of low calcium environment. In the estrogen treated uterus however the depolarisation is maintained and this difference in behaviour has been taken as evidence that progestrone treated uterus either binds calcium more firmly or that more calcium has to be displaced before excitability is lost (28).

Keating (41) using the sheep carotid arteries in a sucrose gap apparatus, showed that after a 30 minute exposure to a calcium free medium a rapid rhythmic electrical activity developed, the onset of which was accompanied by a small slow contraction. Because the arteries never developed a sustained rapid electrical activity even when they were depolarised with potassium sulfate in the presence of calcium,

Keating has concluded that the arterial smooth muscle cell membrane binds calcium unusually strongly.

It has been shown for squid axon, frog myelinated nerve and cardiac muscle that calcium determines the relation between the number of sodium carriers available and the membrane potential (23). An increase in calcium has a similar effect to that of hyperpolarisation, i.e., an increase in the number of sodium carriers available and an increase in the threshold. This may be an adequate explanation for calcium action in the smooth muscle but it does not rule out the possibility that calcium ions might actually carry a part of the inward current during the rising phase of the action potential.

b. Excitation-Contraction Coupling. The coupling process links the excitatory events of the membrane with the chemo-mechanical transducing effected by the contractile protein. From the extensive studies with striated muscle it is postulated the structural site of coupling resides in the endoplasmic reticulum and the functional components of the system include calcium and a relaxing factor. There is little direct evidence that the endoplasmic reticulum and a relaxing factor are involved in coupling in the smooth muscle. Electron microscopic studies indicate that endoplasmic reticulum is poorly developed in the smooth muscle (46), and the efforts to show the presence of a relaxing factor in the uterus have failed (29). The functional role of calcium in the excitation-contraction coupling (E-C Coupling) in the smooth muscle appears to correspond very closely to its role in the same process in the striated muscle. Frank (22) has summarised the

evidence available for involvement of calcium in the E-C Coupling. He has concluded that, a) the introduction of calcium into the muscle fibres can initiate a mechanical response; b) during an action potential or depolarisation there is an increased influx of calcium ions but the information available is insufficient to know if they are entering the fibres in an amount sufficient to initiate contraction; c) the link between electrical and mechanical events in muscular contraction can be severed by removing calcium from the surface of the muscle fibres.

It appears that the excitation-contraction coupling and the role played by calcium ions in this process are basically the same in cardiac and skeletal muscle, but the smooth muscle shows certain differences. Several studies with radioactive isotopes have demonstrated that calcium influxes increase during the contraction of the smooth muscle induced by stimulation. Briggs (10) found that during contractions induced in rabbit aorta strips by potassium sulfate there is a linear relationship between the tension developed and the rate of entry of Ca^{45}. However he was unable to detect a change in the concentration of tissue calcium following such a contraction. He suggested the possibility of increased calcium exchange. This suggestion does not follow his observation that there was no increase in calcium efflux during stimulation.

Using taenia coli of the guinea pig Schatzman (49) could detect no increase in the level of tissue calcium when the muscle was stimulated with acetylcholine or potassium chloride. The efflux of calcium however did increase. He assumed that EDTA titration method was not

sensitive enough to measure the tissue calcium accurately. Sperelakis (53) showed that calcium associated with the activity does not seem to be dependent on the membrane action potential, it is as prominent in the depolarised muscle as it is in the intact muscle. Actually the depolarised muscle is clearly more responsive to elevations in the calcium concentration than is the intact muscle. This suggests that the normal polarised muscle membrane offers an added barrier to the inward movement of calcium ions.

Edman and Schild (17, 18) studied the responsiveness of uterus to acetylcholine during calcium depletion, while being alternately polarised and depolarised in sodium ringer and potassium ringer solutions. They found that polarisation of the membrane potentiated subsequent responses to acetylcholine when the membrane was depolarised. This suggested that the polarised membrane may attract additional calcium to critical sites intracellularly or in the membrane, which is then available for activation by acetylcholine subsequently, when the membrane is depolarised by potassium sulfate. This interpretation utilises the important concept that the stimulating agents, in addition to increasing the calcium influx, are also capable of activating or releasing the bound calcium from these critical sites, and that free calcium ions released are capable of triggering the contractile process. The fact that contractions in response to the stimulating agents persists for a period after the smooth muscle is transferred to a calcium free environment supports the above interpretation. Further evidence for this action of stimulating agent is present in that the repeated

stimulation in a calcium free solution accelerates the decline of the response (17, 18). Each stimulus seems then to add to the exhaustion of the calcium complex.

Waugh (59) using the mesenteric arterial muscle has concluded that calcium is essentially involved in the excitation contraction coupling process in VSM. He showed that injections of isomotic calcium chloride solution of subthreshold or threshold contractile strength, when the artery had been perfused with sodium chloride-rich solution and the muscle had been relaxed, produced much greater contractions during both potassium and adrenaline excitation. The contractions from injected calcium chloride solution were increased by epinephrine excitation of muscle already depolarised by the external application of potassium sulfate, as they were in the previously polarised or resting muscle excited by adrenaline. He suggested that the relative muscular contractions induced by standard injected volumes of isomotic calcium chloride solutions were a relative index of the calcium influx and calcium permeability of the arterial smooth muscle cells in various conditions prevailing in his experiments. He found thus calcium permeability and calcium influx of VSM is increased during both adrenaline and potassium excitation of muscle cells, but adrenaline accomplishes these calcium changes even in muscle already depolarised by potassium. Adrenergic neurohormones he contends exert their vasoexcitatory contractile effects by a membrane reaction which is basically non electrical and which primarily triggers an increased permeability and influx of calcium into the vascular myoplasm. The

migrated calcium activates intracellular myoplasmic events of VSM contraction.

Hinke and Wilson (32) agreed that calcium is essential for muscle contractility, but they did not assume that all stimulating agents made use of calcium in the same way. In isolated arterial segment the contraction in response to noradrenaline and pitressin appears as a maximal contraction between 0.5 and 0.75 mM of calcium. With a potassium contraction the response continues to increase with an increase in concentration of calcium. Hinke (33) has hypothesised that VSM contains at least two calcium fractions, both of which may be bound to the cell surface. One calcium fraction is loosely bound (easily mobilised) and is dependent on the external calcium and the permeability state of the membrane. The other calcium fraction is tightly bound, is dependent on external calcium within relatively narrow limits and may not be too dependent on the permeability state of the membrane. High potassium induced contraction makes use of the loosely bound fraction. The noradrenaline induced contraction on the other hand makes use of tightly bound calcium fraction. Unless external calcium concentration equals zero, noradrenaline may appear also to make use of loosely bound fraction simply because there is always a tendency for calcium to move from loosely bound sites to the tightly bound sites. Furthermore, Hinke (33) has outlined the properties of the tightly bound calcium fraction and the membrane site to which it is bound. Such calcium can not be removed in a calcium free medium but can be removed when EDTA is added. The binding sites are probably

saturated at relatively low amounts of external calcium. The binding of calcium to the site is pH dependent. The binding of calcium to the site is not influenced by external sodium and by a state of polarisation of the membrane. It is also suggested that barium can not substitute for calcium in this site, but it appears to change both the binding capacity and the chemical nature of this site, making it less useful for noradrenaline induced contraction.

This hypothesis appears to be in accordance with the 'Series and Parallel Model' of calcium exchanges across the cell membrane as proposed by Daniel (13). An illustration of this model is given in Figure 14. Daniel has named the loosely bound calcium as superficial calcium, located at sites on the outer side of the membrane, and the firmly bound calcium has been called the sequestered calcium and it is located on the inner side of the membrane. In this model the interstitial calcium reaches sequestered binding sites by a pathway which is by way of the superficial binding sites. In addition, though not essential to such a model, calcium is supposed to be able to reach the cytoplasm by diffusion down its electrochemical gradient through pores, which are not available until calcium is removed from superficial binding sites. As long as the interstitial calcium concentration remains low, these pores will not provide a path for calcium efflux.

Hurwitz (37) using isolated longitudinal muscle from the guinea pig ileum has shown that high levels of calcium ions in the external medium can either inhibit or enhance muscle tone depending upon the experimental conditions that prevail. The maximal responses produced

by the isotonic concentration of potassium chloride were augmented but the submaximal responses produced by 10mM potassium ion were depressed by raising the calcium concentration in the medium from a normal level of 1.8mM to a level of 19.8mM. These findings were explained by Hurwitz in terms of actions of calcium. The concentration of calcium in the bathing fluid was assumed to govern the degree of saturation of the membrane sites with divalent ions. A high degree of calcium saturation provides a large quantity of calcium that can be released or transported inward by an appropriate stimulus. Once released it activates the contractile mechanism. High levels of external calcium ion therefore promote a large increase in tone. But calcium has a second important action at the membrane--a stabilising action. Presumably it antagonises increases in membrane conductance to inorganic ions including its own inward transport and release. Therefore high levels of calcium ions also tend to inhibit an increase in tone. When a large concentration of potassium chloride was used to stimulate the longitudinal muscle the stabilising action of the calcium was largely overcome, a strong activating action of calcium ensued, and a large increase in tone took place. When a small concentration of excitatory agent was employed, the stabilising action was not adequately overcome, and as a result the increase in the smooth muscle was inhibited.

Bohr (7, 8) observed that a vascular smooth muscle response to adrenaline is differentiable into a fast (F) and a slow (S) component. The F component is completed within forty-five to sixty seconds after

the initial stimulation, while the S component persists throughout the remainder of the contraction period. The F component is larger in conditions of low external calcium and is depressed as external calcium increases. The S component on the other hand is usually absent when external calcium is less than 0.3mM and reaches its maximum when calcium concentration is 1mM. He postulated that membrane excitability governs the F component so that as external calcium concentration increases, the membrane is stabilised. On the other hand the rate limiting factor for S component could be the availability of calcium for the coupling process, which is enhanced by increased calcium.

Much consideration has been given to the Lüttagau and Niedergerke (43) hypothesis that sodium competes with calcium for binding sites in the smooth muscle just as it does in the cardiac muscle, and the possibility that the depressing effect of high sodium may be caused by its replacement of calcium at these sites (5, 9, 58). Lüttagau and Niedergerke (43) studied the effects of sodium and calcium ions on the contraction of frog's heart by recording the twitch and potassium chloride contractures of heart strips measuring corresponding action potentials and depolarisations. They confirmed the view that calcium and sodium affect the contractility in an antagonistic way and that the twitch and contracture tensions depend approximately on the ratio: $[Ca^{2+}]:[Na^{+}]^2$ in the bathing fluid. In a detailed analysis of effects they determined an empirical relationship between membrane potentials and contracture tension when the ratio $[Ca^{2+}]:[Na^{+}]^2$ in the bathing fluid was changed to varying extents. Their main findings were that

increasing $[Ca^{2+}]:[Na^{+}]^2$ ratio reduces the amount of depolarisation necessary to obtain a given tension. Furthermore, maximum tension could be obtained even without or with only very little depolarisation when the ratio is greatly increased, i.e., by completely withdrawing sodium at sufficiently large calcium concentrations. These authors concluded that calcium and sodium ions compete at the cell surface or in another cellular region, which is readily accessible to the external solution, for a negatively charged substance to form either a calcium compound activating contraction, or a sodium compound which is inactive:

$$2\ Na^{+} + CaR \rightleftharpoons Na_2R + Ca^{++}$$

This reaction may represent a cation exchange on anionic sites. It may also be interpreted as a chemical reaction involving, for example, the formation of complex calcium and sodium compounds. Taking the total number of anionic groups as unity and assuming that these groups are occupied by either calcium or sodium, the Law of Mass Action leads to:

$$[Ca] / k[Na] = [CaR] / 1 - [CaR]$$

where k, the equilibrium constant of the reaction, is the index of affinity of sodium relative to that of calcium for the anionic site R. The hypothesis explains the facilitating action of lowered sodium as the result of an increased concentration of activating compound CaR, arising from a replacement of combined sodium with extracellular calcium.

It has been reported that an increase in the external sodium is responsible for a decrease in the responsiveness of the smooth muscle (9, 56), and that a decrease in the external sodium causes an increase in the smooth muscle responsiveness in both the resistance and conduit

vessels (4, 36). Hinke and Wilson (32) stated that in the presence of low sodium, contraction of VSM was potentiated during potassium depolarisation but not when noradrenaline was used as the stimulating agent. It was suggested that changes in the contractility induced by varying external sodium appear to act through a sodium-calcium antagonism (5, 58).

Briggs (9) reported that when the sodium chloride concentration in the solution bathing the rabbit aortic strips was reduced Ca^{45} influx in aorta increased by 225% when it was stimulated by adrenaline. In a normal bathing medium the influx increased only 105%. However, using rat uterine muscle Daniel (14) could not demonstrate such an antagonistic effect. He found that shortening of the muscle on elevation of potassium concentration is often enhanced by increasing the sodium concentration while that in response to acetylcholine is reduced. In the uterine muscle sodium prevents relaxation as long as potassium concentration remains elevated. An increase in sodium concentration can produce a contraction of depolarised smooth muscle.

Friedman et al. (24) have observed the activating and potentiating effects of reducing the external sodium concentration and the potentiating effects of reducing the external sodium concentration in both rat colon and VSM. They have proposed that a sodium concentration gradient across the cell membranes may be a primary determinant of the muscle tone and control of the blood pressure.

Neither sodium nor chloride ions appear to be the essential for an adrenergic response. In mesenteric arteries Waugh (58) demonstrated

that a contractile response could be obtained even when sodium chloride had been completely replaced by lithium chloride or sucrose.

c. Protein-Contraction. The contractile protein from uterine, gastrointestinal and vascular smooth muscle has been the subject of many studies. Bohr et al. (6) stated that when these smooth muscle tissues are extracted in a high concentration of potassium chloride (0.5mM) a soluble protein is obtained which has properties similar to those of actomyosin obtained from the skeletal muscle. It forms a viscous solution which undergoes a reversible fall in the viscosity in response to addition of A.T.P. This fall in viscosity is caused by a dissociation of actin from myosin, and is a highly specific characteristic of actomyosin. Its ATPase activity differs from that of actomyosin of skeletal muscle in potency and in dependence on divalent cations and pH, but these dissimilarities can not be interpreted as indicating that the two are basically different. Calcium is a potent activator of the ATPase activity of the actomyosin-like protein obtained from the smooth muscle (6); however, its physiological importance as a direct activator of the chemomechanical transducing effected by the interaction of ATP and actomyosin is not clear.

C. Anions and Smooth Muscle Contraction.

The usual approximate composition of the physiological solutions employed in studies of the response of the smooth muscle is Cl, 120 mEq, HCO_3, 30 mEq and PO_4, 5 mEq. None of these ions appears to be essential for the contractile response of smooth muscle, but the characteristics of the response in VSM can be altered by substitution of other physiological and nonphysiological anions (59). The substitution of bromide for chloride ions in the solution bathing a skeletal muscle results in a marked increase in the twitch tension produced by a single supramaximal stimulus but is without effect on the maximum tetanic tension of the same muscle (40). Other ions of the lyotropic series below chloride in their ability to precipitate proteins from solution are nitrate, iodide and thiocynate (39, 42). Hill and Mcpherson (31) stated that since the maximum tension development and the force velocity relation for the contractile element is unaltered by anions of the lyotropic series it is conceivable that these anions produce their effects on twitch by increasing the duration of activity, so that the contractile element can have more time to shorten and thereby stretch the series elastic component more completely.

The effects on the action potential of various ions used to replace chloride in the external solution have been the subject of a number of reports. Hutter and Noble (38) using the impermeant anion methylsulfate as a substitute for chloride showed that above 17°C the fast phase of repolarisation is not appreciably affected while the negative after potential is increased in size and the duration. An increase

in magnitude and duration of negative after potential has been observed for the anions: Br, NO_3, I, SCN, thiosulfate, monoiodomethane sulfate and acetate by several workers. However, this increased depolarisation does not seem sufficient to account for the increase in the mechanical response, and it has been suggested that the increased mechanical response results from some other action of the anions on the cell membrane (42). That a membrane site is involved in these anion effects is clear from the fact that the effect develops fully before any significant quantities of anions appear inside the cell and that the effects disappear rapidly after long equilibration in solutions containing various anions. The effect of anions on the membrane phenomenon in VSM has not been observed so far.

It has been suggested by Hodgkin and Horowicz (34) from the work of single isolated fibres of the skeletal muscle that since the order in which the anions act: $Cl < Br < NO_3 < I < SCN$ corresponds to their adsorbability the shift of mechanical threshold towards the resting membrane potential might be accounted for by their adsorption at the outer edge of the membrane. Such adsorption of the anions would introduce locally an electric field inside the membrane which subtracts from that contributed by the resting membrane potential. Thus the foreign anions could alter the distribution of other charged groups or dipoles within the membrane without changing the potential difference across the membrane. If a reduction in the electric field at some location within the membrane is the critical event initiating the contraction, such adsorption of anions will shift the membrane potential, which is

just threshold potential for mechanical activation towards the resting membrane potential.

There is evidence that increased influx of calcium ion during depolarisation may represent at least one step of the excitation-contraction coupling (20). Frank (21) using toe muscle of frog investigated the possibility that the effects of anion substitution on contraction might be due to an effect of anions on the calcium entry mechanisms. His results support this concept. Another suggestion concerning the mechanism by which foreign anions potentiate contraction was advanced by Shanes (51). This suggestion rests on the specific hypothesis that calcium entry into skeletal muscle fibres is an important link between excitation and contraction. It is assumed that calcium which is found at certain sites in the membrane is released into the myoplasm as the result of the membrane depolarisation. Shanes (51) suggested that since the foreign anions like Br^-, NO_3^-, I^-, SCN^- and others which are more polarisable than chloride might be expected to increase substantially the binding of calcium in the membrane at rest and enhance the release of calcium into the cell when the membrane is depolarised. This hypothesis appears to provide a basis for the observation of Bianchi and Shanes (3) who found that a 60% increase in active influx of calcium compared with an unchanged "passive influx into the skeletal muscle" during the contraction of skeletal muscle in response to potassium sulfate stimulation.

Reiter (47) studied the effects of various anions on the contractility of the guinea pig papillary muscle and observed that when

NO_3, Br, and I are added to the external medium the rising phase of contraction is prolonged along with an increase in the contractile force. He stated that by the action of these anions the calcium response curve (the dependence of contractile force on calcium concentrations in the medium) is shifted to lower calcium concentrations. This sensitisation to calcium was confirmed by the appearance of after contractions at low temperatures, which have also been found with high calcium concentration and under the action of cardiac glycosides.

Waugh (59) using the contractile response of the perfused dog arterial segments found that isomotic replacement of sodium and chloride in those perfused by sodium iodide, sodium nitrate, sodium bromide and various organic anions markedly potentiated tension responses induced by l-epinephrine. He found that the replacement of chloride with other anions did not contract the quiescent arterial muscle. His findings suggest that the greater contractions resulting from such anions were the result of a greater calcium diffusion across the cell membrane, probably in the form of associated ion pairs of calcium. Also according to Waugh (58, 59) his findings support the suggestion made that transmembranous movements of calcium during the excitation of VSM are significantly accomplished in the form of associated ion pairs of calcium, as appears to be the case in skeletal muscle when NO_3, or Br is substituted for extracellular chloride. Waugh has not suggested what sites of membrane calcium are involved in these movements.

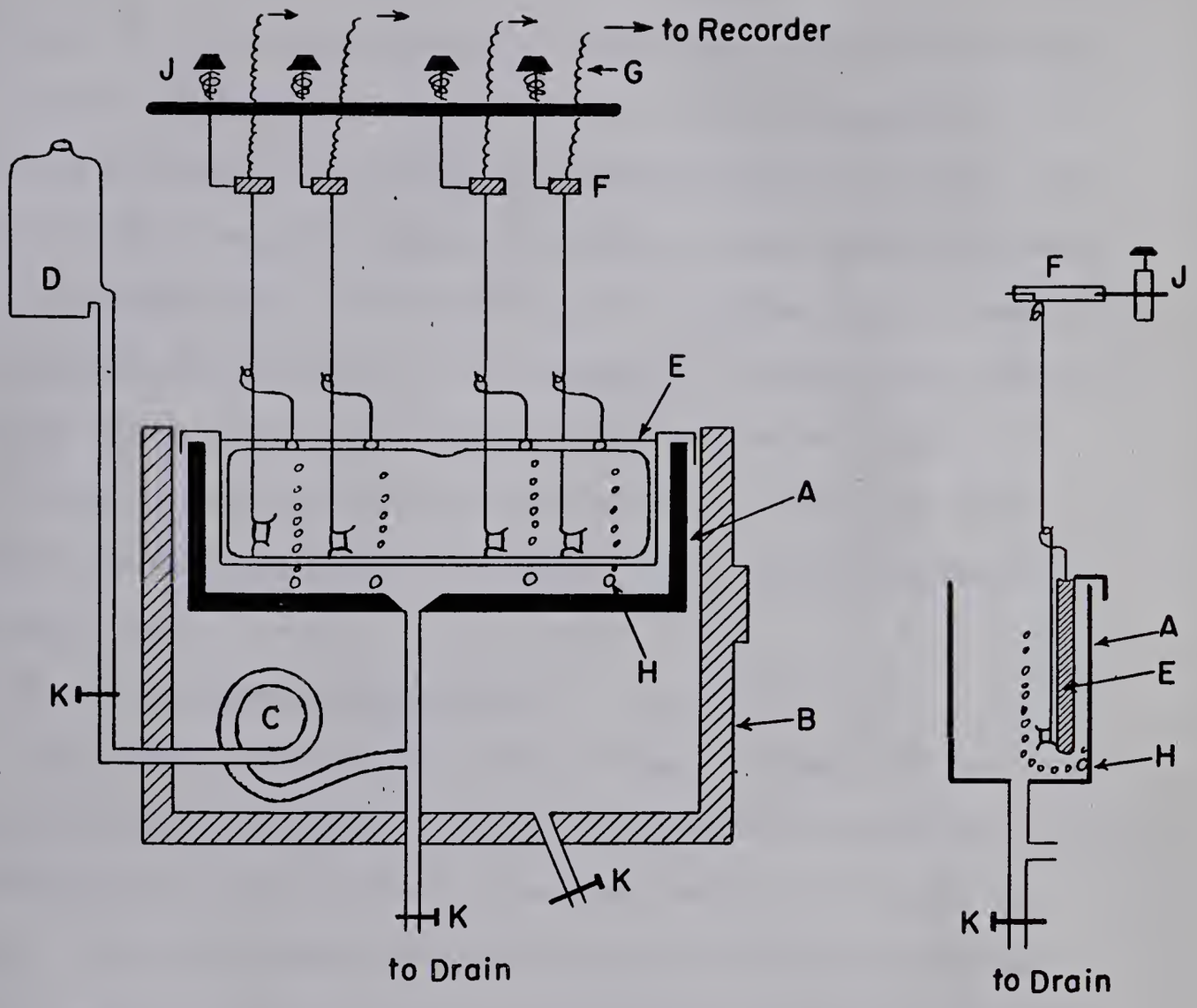
METHODS

Section I. A. Apparatus.

The apparatus is illustrated in Figure 1.

A perspex muscle chamber (A) having the dimensions of 1x5x14 cm. was suspended in a thermostatically controlled water bath (B) maintained at 37°C. An inlet opening at the bottom was connected through the side arm of a glass coil (C) submerged in the water bath and to an overhead reservoir (D), containing the physiological solution used as the bathing medium for the tissues. A second arm of the T tube passed through the bottom of the water bath to act as a drain for the muscle chamber. By opening the pinch cocks on the rubber tubing connections the chamber was filled and emptied manually. To aerate the bath a small bore plastic tubing was fitted into the bottom of tubing and pressure control valves to tanks containing the required gas mixture. Four tissues were suspended on a plastic frame (E) designed to fit into the muscle chamber and having four anchor hooks embedded at its lower edge. The loops of aorta were placed over these hooks and movable stainless steel hooks suspended from upper edge of the frame. The latter were attached to strain gauge force displacement transducers (F) by a second group of stainless steel hooks. Each strain gauge was

FIGURE I



ORGAN BATH AND RECORDING SYSTEM

A) Perspex Muscle Chamber
B) Temperature controlled Palmer Water Bath
C) Glass Coil
D) Reservoir for Solution
E) Perspex Holder (Anchor for Tissues)
F) Strain Gauges
G) Cables
H) Aeration Tubes
J) Baseline Control
K) Pinch Cocks

supported on a screw jack mounted on a metal frame which allowed adjustment of the tension applied to the tissues. The strain gauges were connected to a four channel Grass Polygraph recorder Model 5DWCB, calibrated so that a one gram change in tension produced a 2 cm. deflection on the recording paper. The magnitude of the response could be amplified by increasing the sensitivity of the recorder. The chart speed used was 25mm per second. The record of contractile response was analysed by tabulating the peak deflection for each tissue, at each level of the stimulus and calculating the mean response of all similar tissues at each dose level.

B. Preparation of Aorta Loops.

New Zealand white rabbits weighing between 1.5 and 2 kg. each were killed by a blow on the head. The thoracic aorta was removed as quickly as possible, care being taken to prevent stretching it. Fat and connective tissue were trimmed off and the aorta was cut into loops 3mm wide by means of razor blades held equidistantly apart in a plastic block. Four such loops were suspended in 50 ml. of normal Krebs bicarbonate solution (Appendix I) in the muscle chamber and aerated with 5% CO_2 and 95% O_2 mixture. Two grams tension was applied to each tissue. The muscle loops were allowed to relax for 2 or 3 hours after suspension in the organ bath with the tension maintained at 2 grams. After the responses of the tissue to a stimulus had become reproducible experimental observations were begun.

C. Methods of Stimulation.

The l-noradrenaline bitartrate(NA) used to stimulate the tissues

was prepared as a stock solution containing 1.0 mg. NA per ml in 0.01 N hydrochloric acid. The stock solution was further diluted with isotonic saline to prepare the desired concentration of NA for stimulation of the tissues. The drug was left in contact with the tissues for 3 minutes before the bath drained and refilled with fresh electrolyte solution. The bath was drained and refilled with fresh electrolyte solution every six minutes. The interval between successive test stimulations was kept constant for any one experiment and was determined by the time required for a complete relaxation of the tissues from the previous response, but in no case was it less than 15 minutes. A 0.8 molar potassium sulfate solution used for stimulation was prepared by dissolving the solid in deionised distilled water and kept in solution by maintaining the temperature at $37^{o}C$. The concentration of potassium in the bathing medium was raised by injecting into the bath appropriate amounts of this solution to form the desired potassium ion concentration (see results). The time course and the dose frequency of potassium stimulation was the same as that for NA stimulation (see above). The electrical stimulation applied was 8 or 12 volts, 60 cycles A.C., adjusted with a variable transformer (Variac) for a duration of 5 seconds.

Section II. Ionic Alterations and Contractility.

A. Effect of Changes in CO_2 and Calcium Concentration.

The organ bath was filled with Krebs bicarbonate solution which was aerated with a mixture of 5% CO_2 and 95% O_2. To determine the influence of calcium present in the tissues on the response of the tissues, a contractile response to an electrical stimulus was recorded and the stimulation was repeated until the response became constant. To remove calcium the tissues were exposed to calcium free Krebs bicarbonate solution containing a 4mM concentration of disodium salt of ethylene diamine tetracetic acid (EDTA). Finally EDTA was removed by two more 6 minute washes in calcium free Krebs bicarbonate solution. Rabbit aorta tissues respond to electrical stimulus after exposure to calcium free solution for several hours but after treatment with EDTA the response is almost abolished. After the tissues were made free of calcium, they were exposed for 18 minutes to Krebs bicarbonate solution containing the desired amount of calcium (see results, Figure 2A) and were again tested for their response.

To determine the effects of a high concentration of CO_2 on the response induced by electrical stimulation, a control response was first recorded in the normal bathing medium with 5% CO_2. The tissues were then exposed to 30% CO_2 for 6 minutes and stimulated. Fifteen minutes later when the recovery was complete the control response with 5% CO_2 was reproduced. The effect of isopropylnoradrenaline (INA) on the response of the tissues was determined in a similar way, the tissues

being exposed to INA for 6 minutes before stimulation. The effect of a β-adrenergic blocking agent dichloroisoproterenol (DCI) on the changes in the response produced by INA and 30% CO_2 was determined by injecting a dose of DCI in the organ bath before INA or 30% CO_2 were introduced.

B. Sodium and Calcium Competition in VSM.

The investigation of sodium-calcium competition for active sites in VSM was divided into three sections:

1) Determination of the sensitive range of calcium concentration for contraction of the tissues.
2) Tests of sodium and calcium competition in response to NA, to electrical and potassium stimulation.
3) Tests for a change in response due to the increase in the ratio of sodium ions inside to outside the muscle cell, i.e., $[Na^+]_i : [Na^+]_o$.

The range in which changes in calcium concentration resulted in changes in the response to a fixed stimulus was determined by preparing the calcium free tissues as described on page 25, and testing for the responses to two voltage levels (8 and 12 volts) or two dosage levels of NA (10^{-7} and 5×10^{-7} grams per ml) at each of a series of increasing concentrations of calcium in the Krebs bicarbonate solution used as the medium.

In the experiments designed to test for sodium-calcium competition the effects of changes in calcium concentration in the media of normal (143mM) and reduced (71.5mM) sodium concentration were studied. As in the previous series, the aorta loops were pretreated with a calcium-free

medium containing EDTA. When the tests were to be carried out in the sodium deficient medium the pretreatment medium contained the desired amount of calcium but was also made sodium deficient. The total pretreatment exposure to low sodium was 18 minutes. Whether the medium contained normal or low amounts of sodium the order of testing was from lowest to the highest calcium concentration (i.e., from 0.08mM to 2.5mM). Stimulation was either by NA, electric current or excess potassium. The tension developed was plotted against the ratio of $[Ca^{2+}] : [Na^{+}]^2$.

Tests in which the ratio $[Na^{+}]_i : [Na^{+}]_o$ was increased were carried out by exposing the tissues to a low Na Krebs bicarbonate solution allowing only one minute for equilibration before stimulation. In these experiments the tissues were pretreated with EDTA in the usual way, immersed in Krebs bicarbonate solution containing 143mM sodium and 1/8 normal calcium, and then stimulated with NA. The solution was then changed to Krebs bicarbonate solution containing 71.5mM sodium and 1/8 normal calcium. The response to NA was again tested exactly one minute after changing the bath fluid. As the results showed a potentiation of response after a brief exposure to low Na solution, an experiment similar to that described above was performed to test for calcium and sodium competition, but in this case the time of exposure to solution containing a low Na was limited to 1 minute before stimulation of the tissue.

C. Effects of Inorganic Anions on Contractility of VSM.

In the investigation of the effects of anions on the contractility

of VSM a series of modified Krebs bicarbonate solutions were prepared. In these solutions sodium chloride was replaced by an equivalent amount of another sodium salt. The anions used were: bromide, nitrate, iodide, thiocynate and sulfate, as their sodium salts. The methods of stimulation were: noradrenaline, excess potassium and electrical current.

The response of tissues to the required doses of NA were recorded at first in the normal Krebs bicarbonate solution. A dose of NA was selected and its response was reproduced under the same conditions. When the tissues had recovered from contraction, they were exposed to the solution containing a foreign anion for one minute, and stimulated with the same dose of NA. The drug was left in contact with the tissues for three minutes before the bath was drained and refilled with fresh modified Krebs bicarbonate solution ('Anion Krebs solution'). The exposure to the foreign anion was continued for 12 minutes (2 x 6 minute washes) and the tissues were stimulated again with the same dose of NA. Similarly using different doses of NA, and the time of exposures of 1 minute and 15 minutes to 'Anion Krebs solution', the contractile responses of the tissues were recorded. The experiments were repeated using various anions.

In a subsequent series of experiments performed using all the anions mentioned above, potassium sulfate and electrical current (8-12 volts for 5 seconds) were used as the stimulating agents.

The Standard Errors of the means of the responses at each dose were calculated using the following formula:

$$SE = \sqrt{\frac{\Sigma X^2 - (\Sigma X/n)^2}{n(n-1)}}$$

The Students Paired t-test was used to calculate the significance of the difference between the two points which were being compared:

$$t = \frac{\Sigma(X_1 - X_2)}{n} \Bigg/ \sqrt{\frac{\Sigma(X_1 - X_2)^2 - \frac{(\Sigma X_1 - \Sigma X_2)^2}{n}}{n(n-1)}}$$

P values were obtained from Fisher's distribution tables.

RESULTS

A. Effects of Changes in CO_2 and Calcium Concentration.

In normal Krebs bicarbonate solution the contractile response of the isolated rabbit aorta loops stimulated electrically consists of a dual response. The tissues give a rapid initial (fast) response lasting 10 to 15 seconds and a more prolonged (slow) response which lasts well over two minutes. Figures 2A and 2B show that, on depletion of tissue calcium by treatments with calcium-free Krebs bicarbonate solution and EDTA, the contractile response of the tissue was reduced but not eliminated, and that the size of the delayed response was reduced more than that of the initial response. When the calcium concentration in the bath medium was progressively increased from 0 to 2.5 mM, the contractile response increased correspondingly. Plotting the initial and delayed responses against the calcium concentration (Figure 2B) showed that the slow response is more dependent on the external calcium concentration than the fast response.

Figure 3 shows that the increase in CO_2 from 5% to 30% in the gas mixture aerating the normal Krebs bicarbonate solution reduced the slow response and that INA in a concentration of 2 μg/ml produced the same result. Figure 4 shows that DCI, a β-receptor adrenergic

blocking agent which itself had no effect on the response, prevented the depressive effect of INA on the delayed response. In the same concentration DCI had no effect on the depression produced by 30% CO_2. It therefore appears that the depressive effect of CO_2 on vascular smooth muscle is not effected through β-adrenergic receptor sites in the tissue. Figure 5 shows the initial responses are much greater than the delayed responses of tissues previously treated with dibenzyline, an adrenergic receptor blocking agent, and of tissues obtained from rabbits previously treated with reserpine. A high concentration of CO_2 did not reduce the initial response but it did reduce the delayed response.

B. Sodium and Calcium Competition in VSM.

The effect of changes in the external calcium on the response of the aorta loops to NA and to electrical excitation are shown in Figure 6. In both experiments with NA and with electrical stimulation the maximum responses were elicited at 1/4 to 1/2 of the calcium concentration present in normal Krebs bicarbonate solution. An experiment designed to test for a calcium and sodium competition in the VSM should therefore be done at a calcium concentration range in which a small change in calcium can elicit a definite change in the response to applied stimulus. The subsequent tests were made with calcium concentration between 1/16 and 1/4 of the normal concentration in the Krebs bicarbonate solution.

The results of tests for sodium and calcium competition are shown

in Figures 7A and 7C, where the tension developed is plotted against the ratio of $[Ca^{2+}] : [Na^{+}]^2$. The response increased in the media, both low and normal in sodium, as the calcium concentration was increased. In low sodium solutions, even though the $[Ca^{2+}] : [Na^{+}]^2$ ratios were always greater than in solutions containing normal amounts of sodium, the responses obtained were considerably less. In fact when the $[Na^{+}]$ was reduced to half normal, thus increasing the ratio $[Ca^{2+}] : [Na^{+}]^2$, the mean response was unchanged in one test, reduced in one test, and slightly increased in two tests. When electrical stimulation was used the responses were reduced in all tests when the [Na] was reduced. The two solid lines in each of the sections of Figure 7 emphasize that there was no evidence of the responses falling along a common regression line.

The results of the tests for a change in response due to alteration in ratio of $[Na^{+}]_i : [Na^{+}]_o$ are shown in Figure 8. At low dosage levels of NA there was a significant increase in the response during exposure to a low $[Na^{+}]$ for 1 minute, but this difference disappeared as the dose of NA was increased. In Figure 8 the data shown in Table 4 have been plotted as double reciprocal curves to determine whether the maximum response was altered in low sodium environment. The regression lines have approximately the same intercept on the origin, indicating that the treatment did not influence the maximum response. A test for sodium and calcium competition was applied, in which the time of exposure to solutions containing low sodium was limited to one minute before stimulation of the tissues. The results are shown in Figures 7B and 7D. Plots of response against activity ratio $[Ca^{2+}] : [Na^{+}]^2$

show that the response of the tissues in normal $[Na^+]$ and in reduced $[Na^+]$ fall on separate curves. Thus any single value of the activity ratio $[Ca^{2+}] : [Na^+]^2$ (e.g., in Figures 7A and 7C) shows two distinct responses, their sizes depending on Ca. When one minute exposures to reduced Na were used the responses produced by either drug or electrical stimulation were greater than those occurring at normal Na. But when exposure to reduced Na was extended to 18 minutes (Figure 7) the responses in reduced Na were smaller than those in normal sodium.

In all this work there is a possibility that the stimulation was not constant under the two different electrolyte conditions. Therefore in addition to those reported above a series of experiments was done with potassium sulfate (a solution of potassium sulfate added to 50 ml. of Krebs solution in an organ bath, thus raising potassium concentration to 54mM) used as the stimulating agent. Results of the tests indicated that depolarisation with potassium sulfate resulted in very uniform contractile response of these tissues, and the results of these experiments, shown in Figure 9, were very similar to those reported above. Although it does not exclude the possibility of a variation in stimulation with altered conditions, the fact that three different methods of stimulation gave very similar results considerably reduces the probability of error arising for that reason.

C. Effect of Inorganic Anions on Contractility of VSM.

In experiments in which chloride ions of the normal Krebs solution are substituted with a foreign anion of the series Br, NO_3, I, and SCN

or SO_4, the contractile response obtained varied with the anion present and the type of stimulus used. The effects of these substitutions, using three types of stimuli, potassium sulfate, noradrenaline, and electrical, are shown in Figures 10, 11, 12 respectively. The responses in the normal Krebs bicarbonate solution were used as controls, and in order to demonstrate the degree of potentiation or inhibition caused by anion substitution the responses in the modified solutions were expressed as the percentage of the control response.

Figure 10 shows the results of experiments in which potassium sulfate was used as the stimulating agent in concentration 8, 16, 32, and 64mM. It was found that Br, NO_3, I, and SCN all potentiated the contractile response of the tissues, the order of potency being SCN > I > NO_3 > Br > Cl. The degree of potentiation increased as the time of exposure to the anion before stimulation was prolonged from one minute to 15 minutes. As the concentrations of potassium sulfate were progressively raised the degree of potentiation diminished correspondingly.

Figure 11 shows the results of experiments in which the tissues were stimulated with noradrenaline in doses, 0.1, 0.2, 0.4, and 1.0 μg/ml in the external medium. It was found that whereas Br and NO_3 produced a potentiation of the contractile response, the ions I and SCN produced an inhibition under the same conditions. The order of potency in which these ions ranked was NO_3 > Br > Cl > SCN > I. The prolongation of exposure time from 1 minute to 15 minutes increased the response produced in the presence of Br, NO_3, I and SCN, although after 15 minutes exposure the response in presence of I and SCN was still inhibitory. As in the

case of stimulation with potassium the modification of the contractile response was greater at lower dose levels than at higher dose levels.

The electrical stimulus, 8 and 12 volts for 5 seconds, was used primarily to observe the effect of foreign anions on two phases of the response induced by this stimulus. Figure 12 shows the results of these experiments. It was found that the contractility of the tissues was diminished in the presence of Br, NO_3, I, and SCN regardless of the strength of the stimulus, or the time of exposure to the anion prior to stimulation. A satisfactory analysis of the action of the anions on two phases could not be made because the recovery of the muscle was adversely affected and the tissues could not be stimulated to reproduce the control responses.

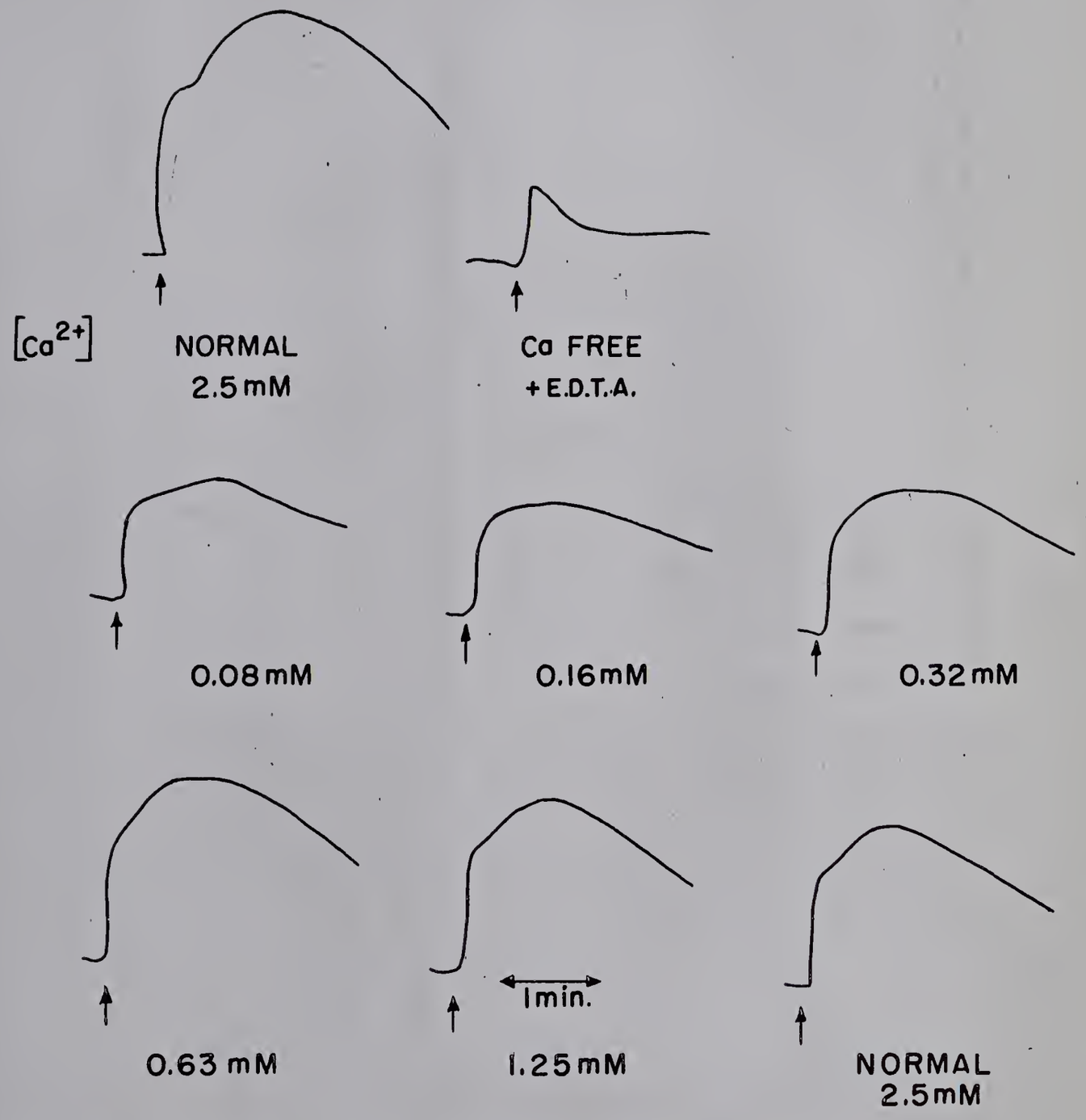
Figure 13 shows the responses of the tissues to potassium, noradrenaline and electrical stimulation in a solution where the chloride ions were substituted with sulfate. The response under these conditions was an inhibition of the contractile response regardless of the stimulus or the time of exposure. However the inhibition was by far greatest in the case of tissues stimulated with potassium sulfate (over 50%). The inhibitory effect was much less on the responses evoked by noradrenaline (less than 20%).

Figure 2A.

The Effect of Calcium on Response of VSM to Electrical Stimulation.

The contractile responses to a fixed electrical stimulus (12V-5Sec. a.c.) were obtained in normal Krebs bicarbonate solution containing varying amounts of calcium. For further details see Figure 2B.

FIGURE 2A



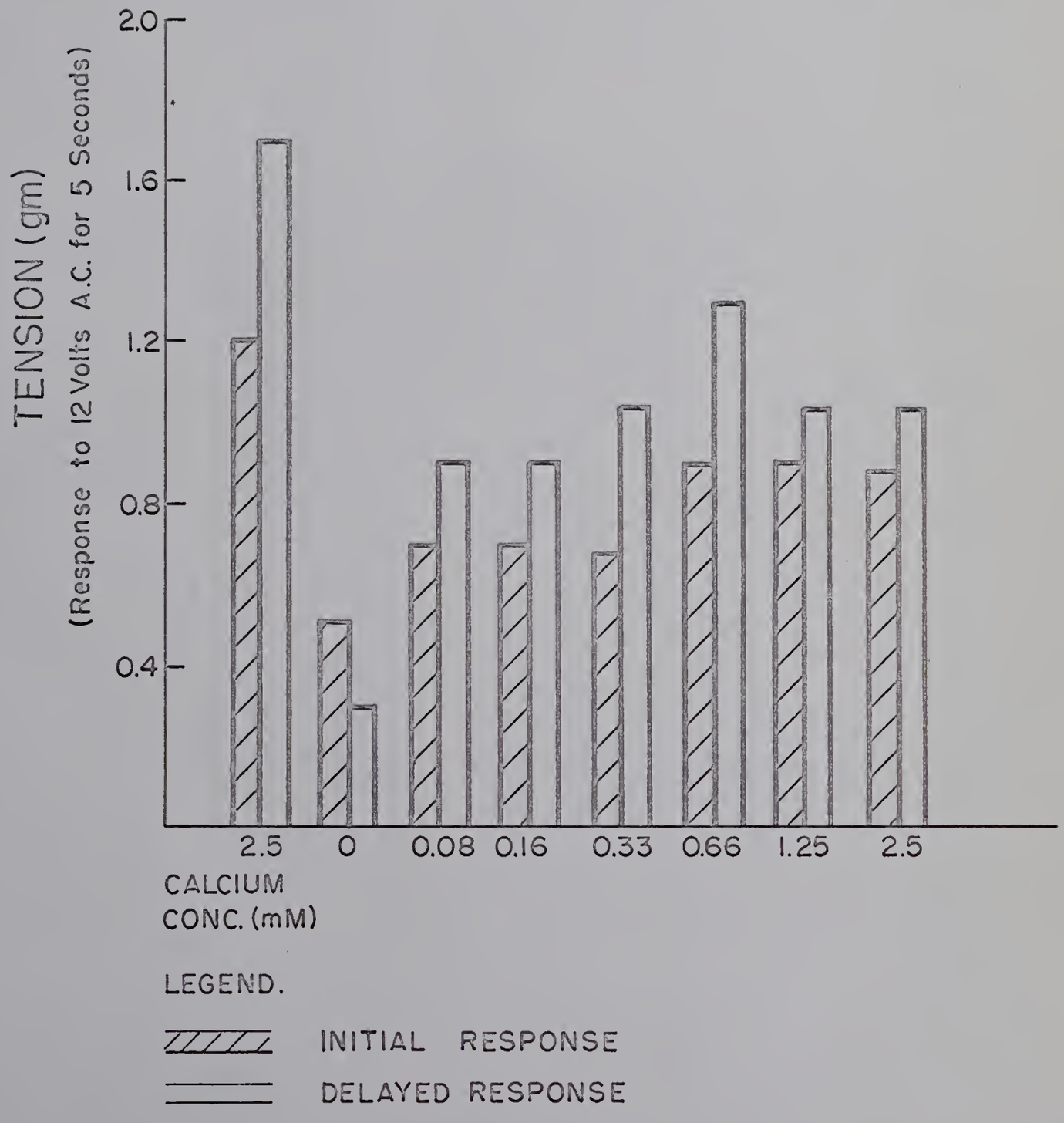
↑ ELECTRICAL STIMULUS. 12 VOLTS - 5 SECONDS

Figure 2B.

The Influence of Calcium Concentrations on the Dual Response of Vascular Smooth Muscle to Electrical Stimulation.

The contractile responses of rabbit aorta tissues in normal Krebs bicarbonate solution containing varying amounts of calcium is shown. The initial portion of the response was measured from baseline to the flexion point (see Figure 2A). The delayed portion of the response was taken as the tension remaining one minute after stimulation. The delayed response increased in magnitude as calcium was increased from 0 to 0.66 mM. The corresponding data are found in Appendix II, Table I.

FIGURE 2B


TENSION (gm)
(Response to 12 Volts A.C. for 5 Seconds)
2.0
1.6
1.2
0.8
0.4
2.5
0
0.08
0.16
0.33
0.66
1.25
2.5
CALCIUM
CONC. (mM)
LEGEND.
INITIAL RESPONSE
DELAYED RESPONSE

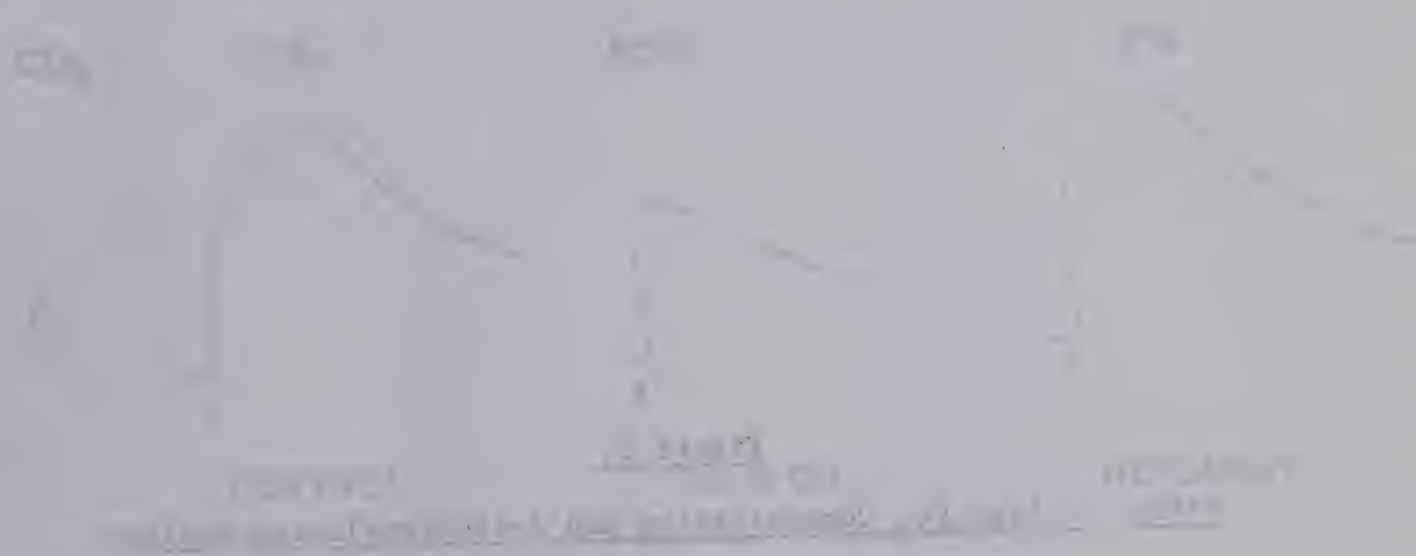
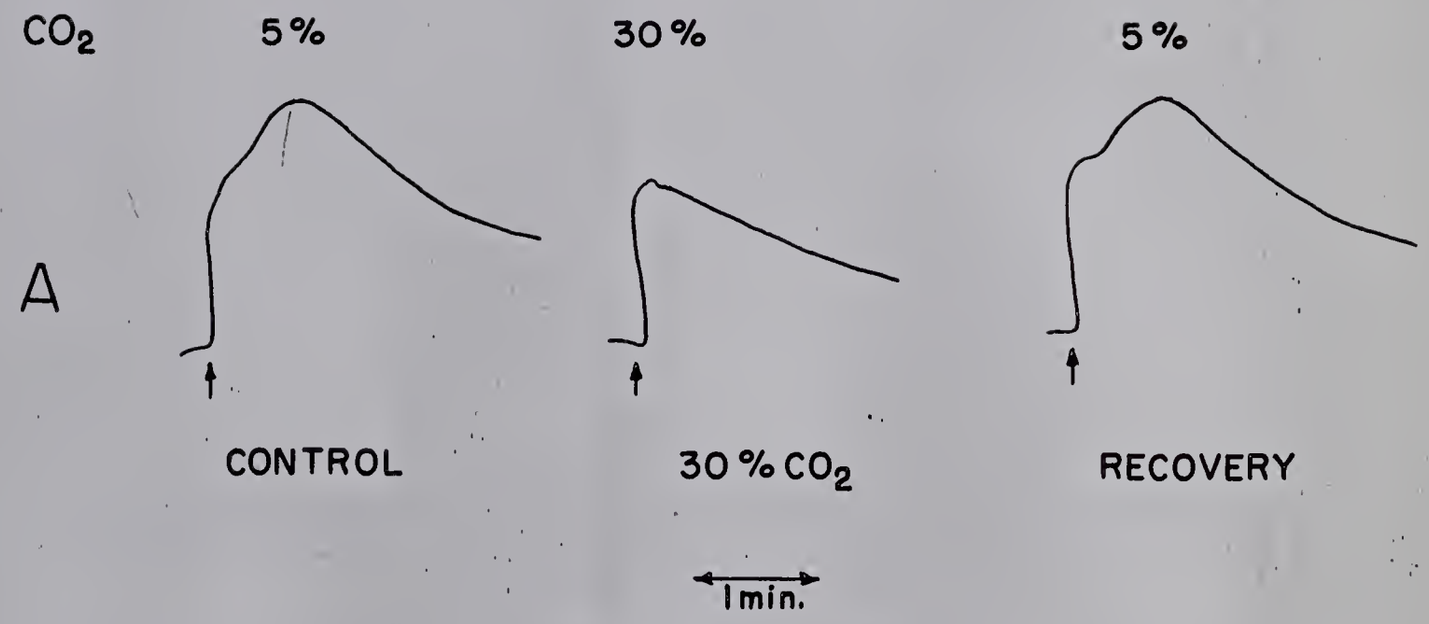


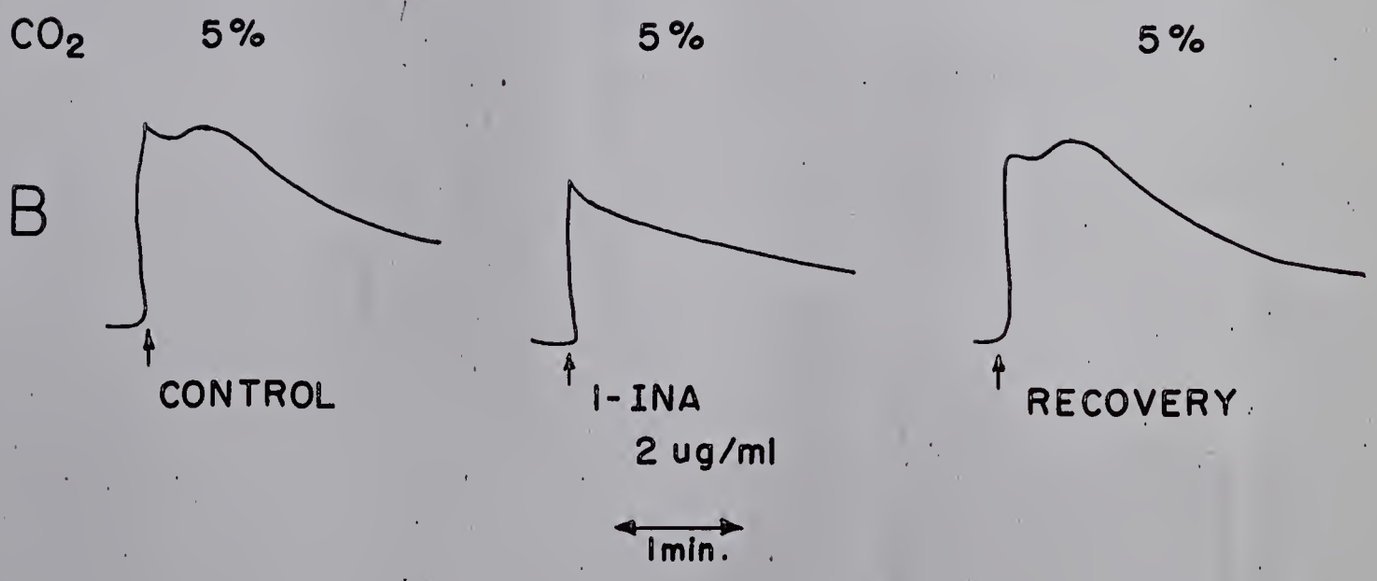
Figure 3.

Effect of High CO_2 Concentration and 1-isopropylnoradrenaline on the Slow Phase of the Response of VSM to Electrical Stimulation.

Both high concentration of CO_2 and 1-INA (2μg/ml) abolished completely and reversibly the slow phase of the biphasic response. These agents had little or no effect on the fast phase.

FIGURE 3





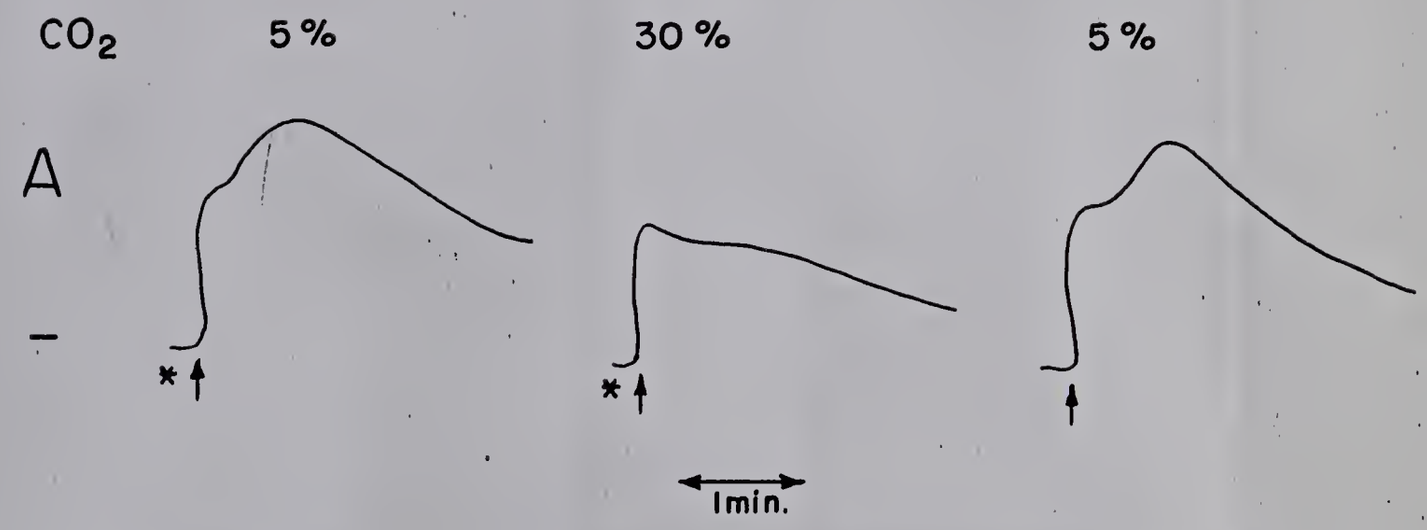
↑ 12 VOLTS - 5 SECONDS

Figure 4.

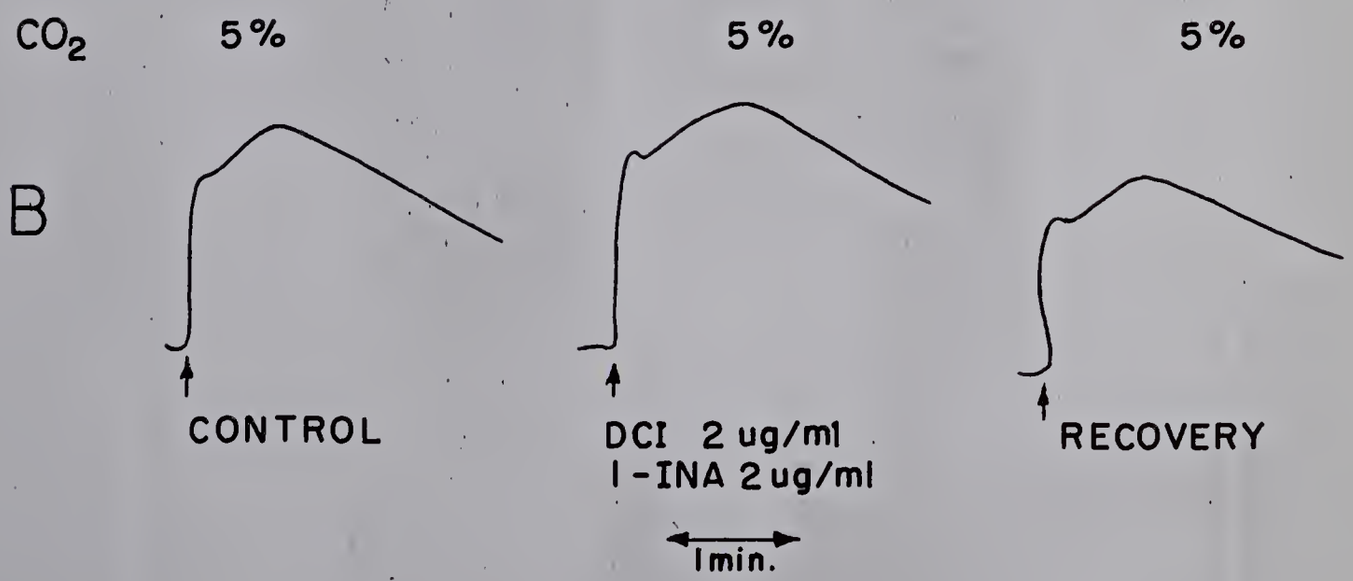
Effects of Dichloroisoproterenol on the Depressive Action of High CO_2 and l-INA in VSM.

DCI (2μg/ml) by itself had no effect on the response of the tissues to electrical stimulation and it did not affect the depression produced by 30% CO_2. In the same concentration DCI abolished the depressant effect of l-INA on the slow phase.

FIGURE 4



* D.C.I.– 2 ug/ml



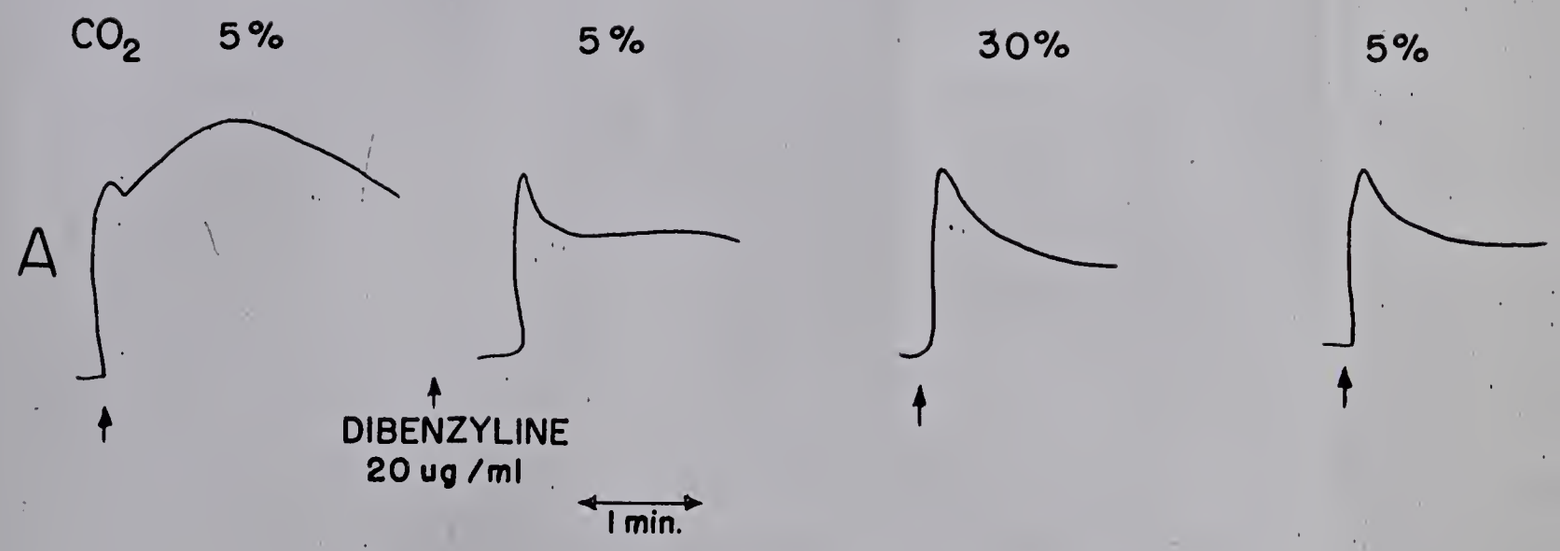
↑ 12 VOLTS – 5 SECONDS

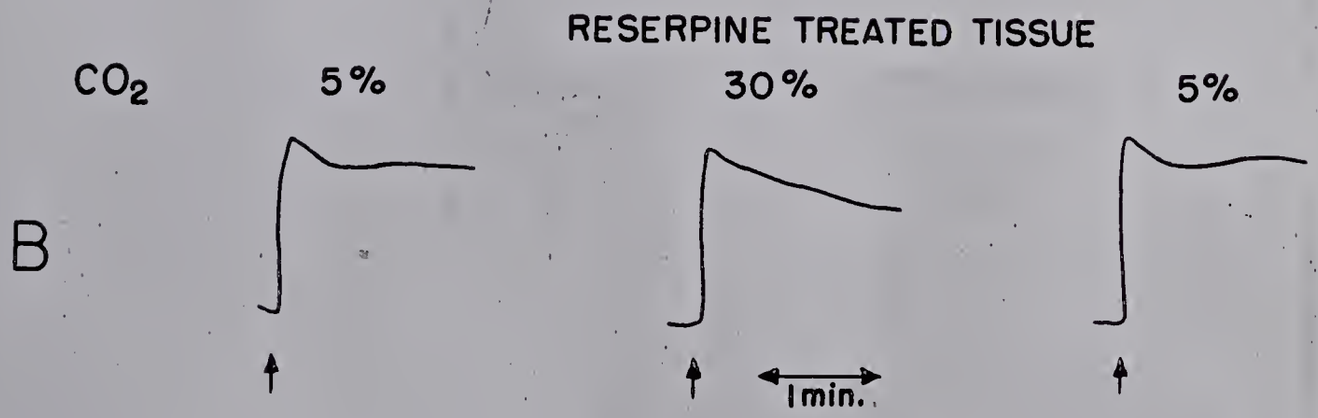
Figure 5.

Effect of High CO_2 on the Fast Phase of the Response of VSM in Tissues Pretreated with Dibenzyline and Reserpine.

In the tissues treated with dibenzyline (20μg/ml) and the tissues from reserpine treated rabbits (2.5mg/kg for 2 days), the response consists mainly of the fast phase. In both cases 30% CO_2 failed to depress the fast phase of the response.

FIGURE 5





↑ 12 VOLTS - 5 SECONDS

Figure 6.

Influence of Calcium Concentrations on the Responses of VSM to Noradrenaline and Electrical Stimulation.

In each experiment the mean response (four to eight tissues) to the higher of the two test stimuli, before calcium depletion, was taken as 100%. The corresponding data are found in Appendix II and Table 2.

FIGURE 6

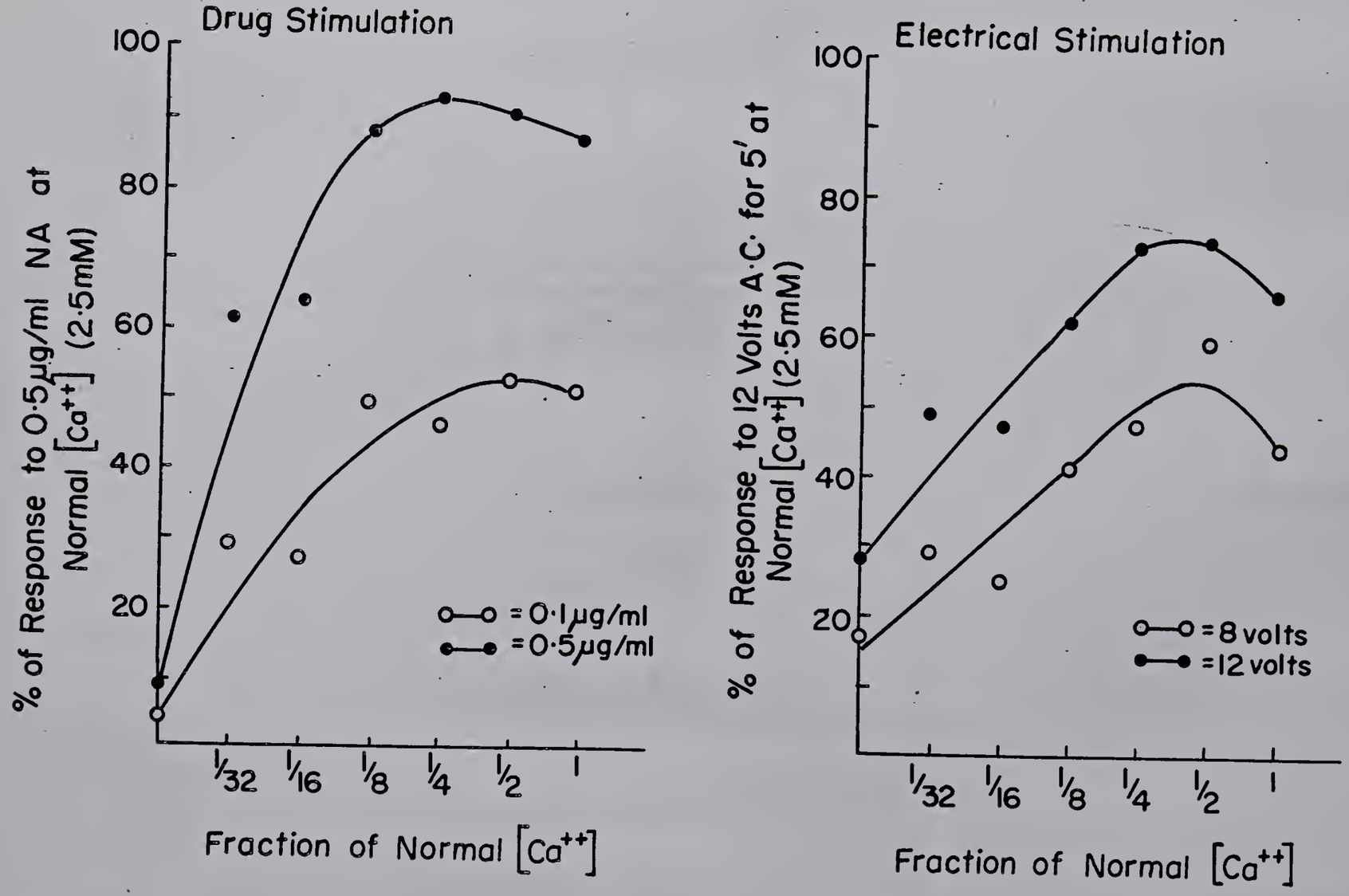

Drug Stimulation
% of Response to 0·5µg/ml NA at Normal [Ca++] (2·5mM)
100
80
60
40
20
= 0·1µg/ml
= 0·5µg/ml
1/32
1/16
1/8
1/4
1/2
1
Fraction of Normal [Ca++]
Electrical Stimulation
% of Response to 12 Volts A·C· for 5′ at Normal [Ca++] (2·5mM)
100
80
60
40
20
= 8 volts
= 12 volts
1/32
1/16
1/8
1/4
1/2
1
Fraction of Normal [Ca++]

Figure 7.

Tests for Evidence of Sodium and Calcium

Competition in VSM.

The contractile response to fixed stimuli (0.25 μg/ml NA and 12 volts a.c. for 5 seconds) were plotted against activity ratios for the concentration of sodium and calcium in the bath. Each point is a mean of 8-10 variables. The broken lines join same calcium level but with half normal sodium concentration. The slopes of broken lines indicate the direction of change in response. As indicated by solid lines in each of the four experiments the responses fell on separate regression lines depending on sodium concentration, thus showing no evidence that sodium and calcium competition controls the response to the stimulus. Exposure to reduced $[Na^+]$: A and C, 18 minutes, B and D 1 minute. The corresponding data are found in Appendix II, Table 3.

FIGURE 7

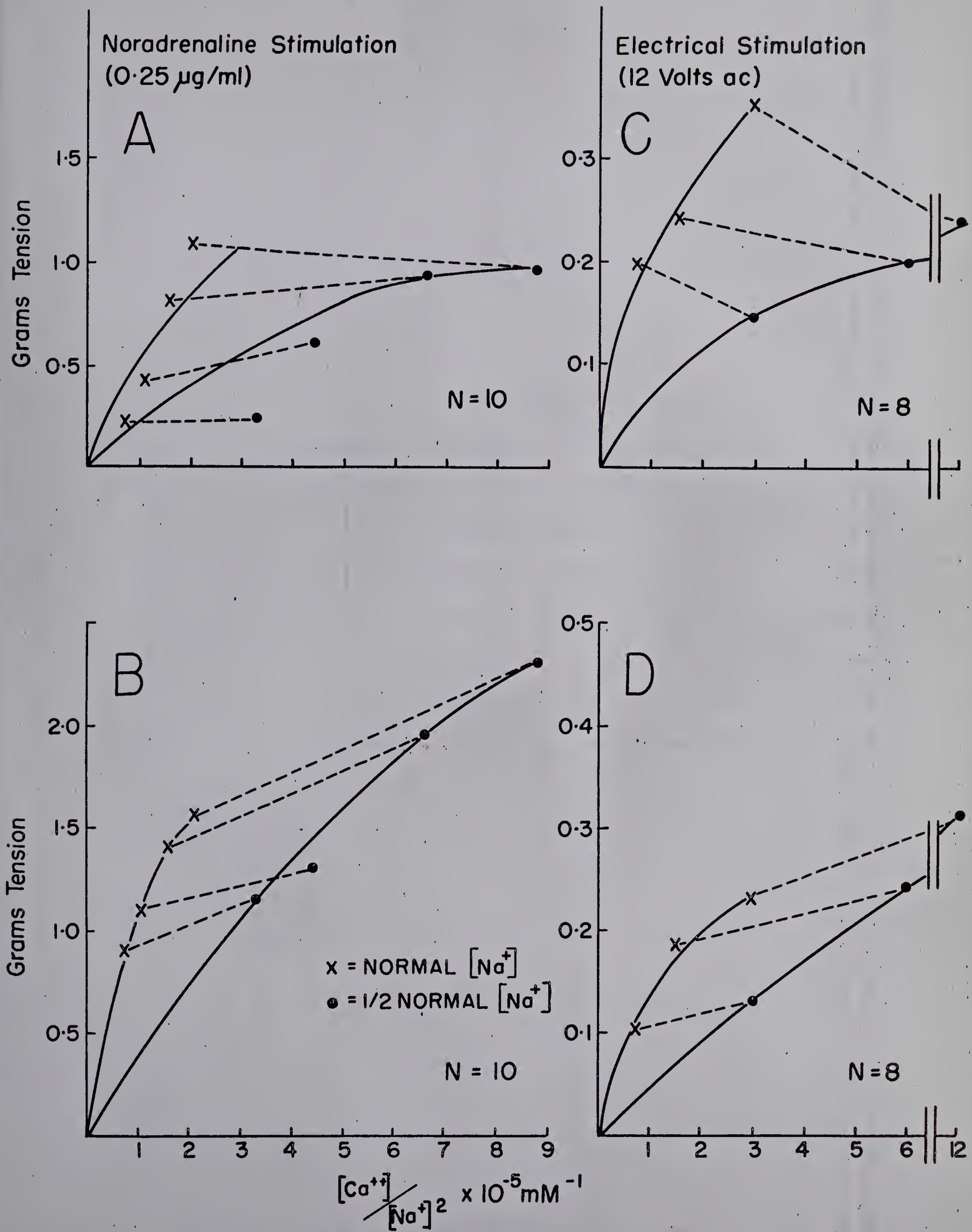
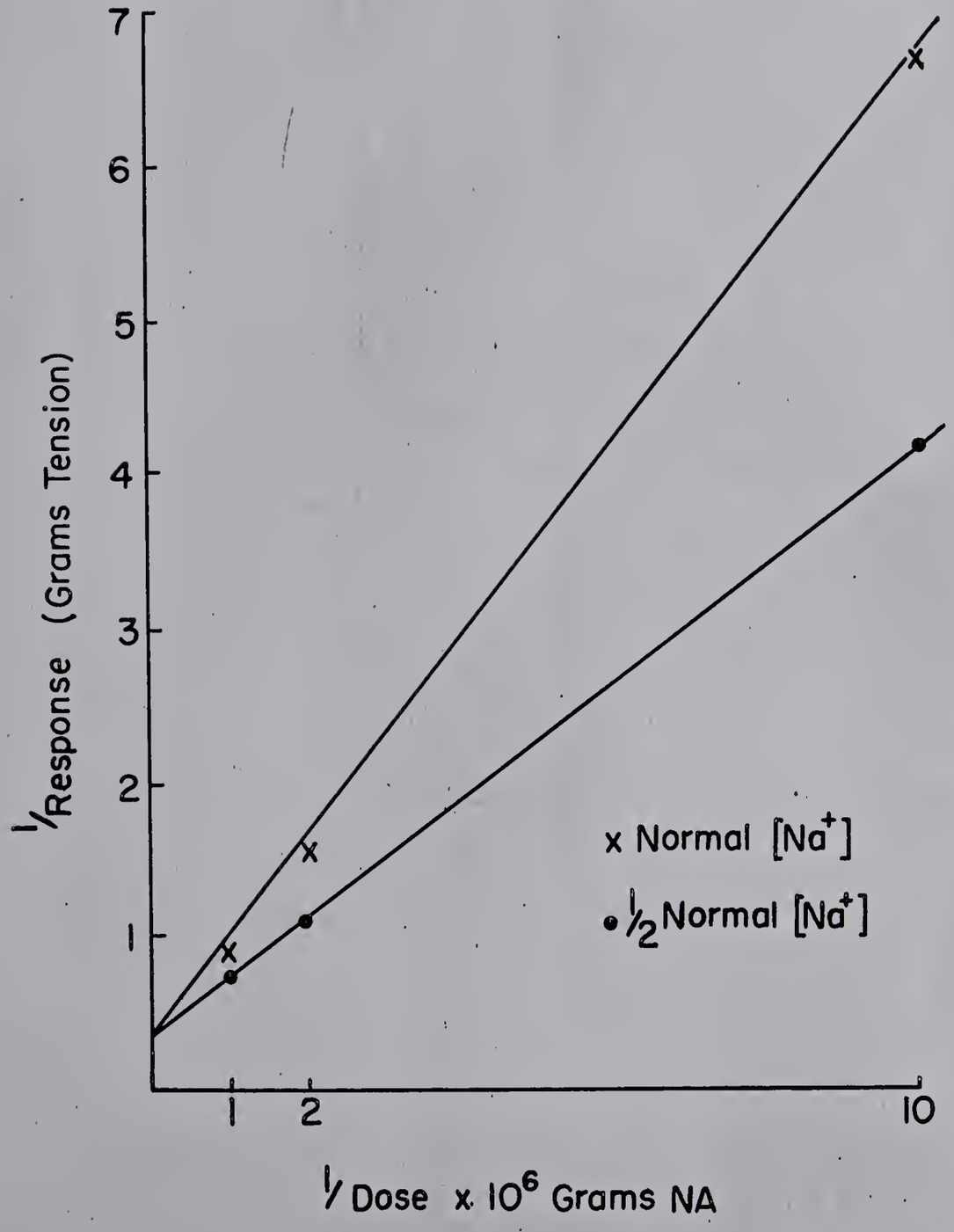

Noradrenaline Stimulation
(0·25 µg/ml)
A
Grams Tension
1·5
1·0
0·5
N = 10
Electrical Stimulation
(12 Volts ac)
C
0·3
0·2
0·1
N = 8
B
2·0
1·5
1·0
0·5
x = NORMAL [Na+]
● = 1/2 NORMAL [Na+]
N = 10
1 2 3 4 5 6 7 8 9
[Ca++] / [Na+]2 x 10-5 mM-1
D
0·5
0·4
0·3
0·2
0·1
N = 8
1 2 3 4 5 6 12

Figure 8.

Effects of Low Sodium Concentrations on the Contractile Responses of VSM to Noradrenaline.

Tissues were bathed in modified Krebs bicarbonate solution containing 1/8 normal Ca, and normal Na or half normal Na. The latter was applied for 1 minute before the stimulating dose of noradrenaline. Each value is a mean of responses of 10 separate tissues. The double reciprocal plot indicates that the maximum response to noradrenaline was not altered by reduction of sodium content of the medium. Submaximal responses were substantially increased at low dosage levels. The corresponding data are found in Appendix II, Table 4.

FIGURE 8


7
6
5
4
3
2
1
1/Response (Grams Tension)
x Normal [Na+]
• 1/2 Normal [Na+]
1
2
10
1/ Dose x 10^6 Grams NA

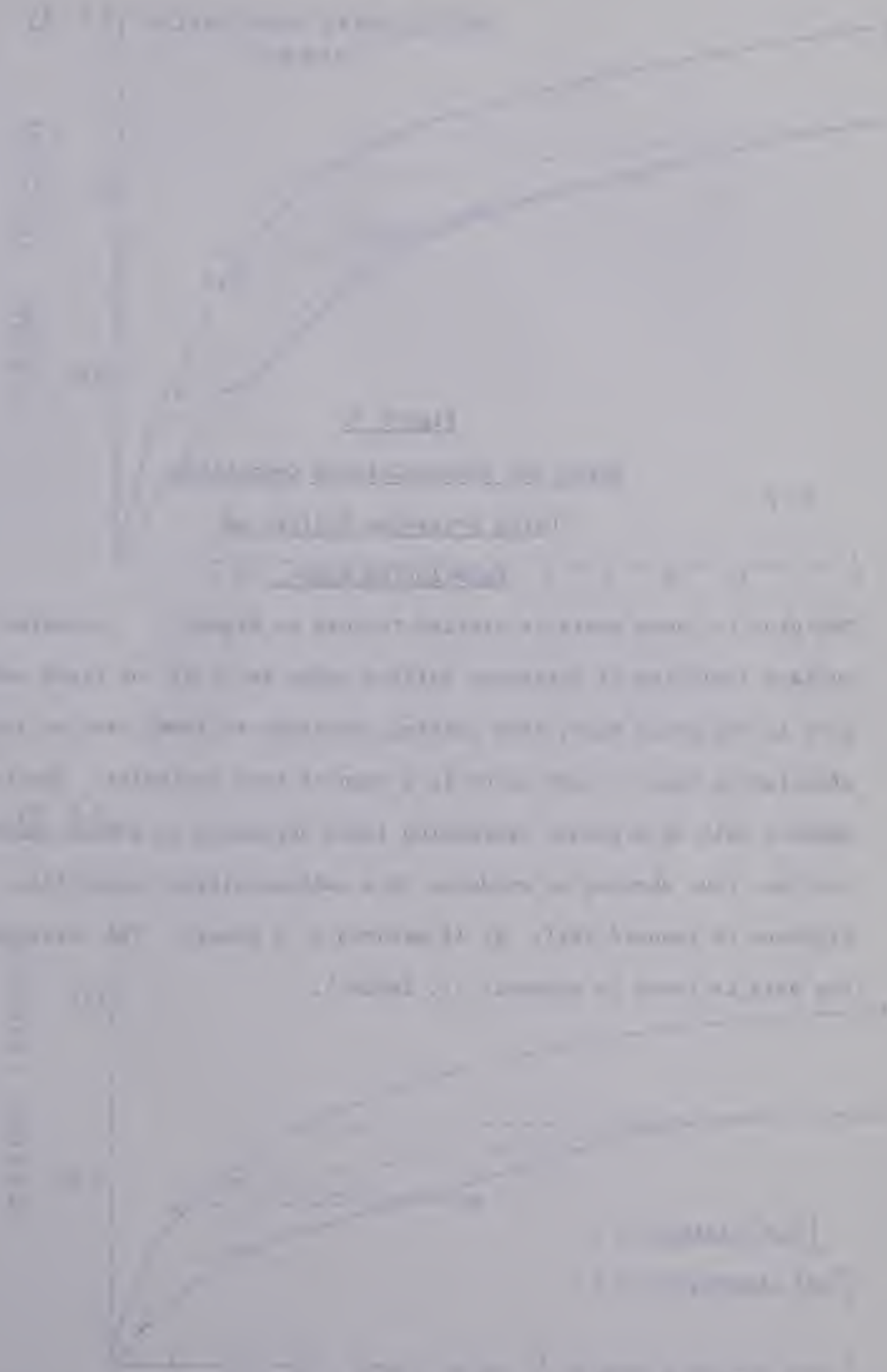


Figure 9.

Tests for Sodium-Calcium Competition

Using Potassium Sulfate as

Stimulating Agent.

The plot in these tests is similar to that in Figure 7. Potassium sulfate (solution of potassium sulfate added to 50 ml. of Krebs solution in the organ bath, thus raising potassium to 54mM) used as the stimulating agent. Each point is a mean of four variables. The responses fell on separate regression lines depending on sodium concentration, thus showing no evidence of a sodium-calcium competition. Exposure to reduced [Na]: A. 18 minutes B. 1 minute. The corresponding data is found in Appendix II, Table 5.

FIGURE 9

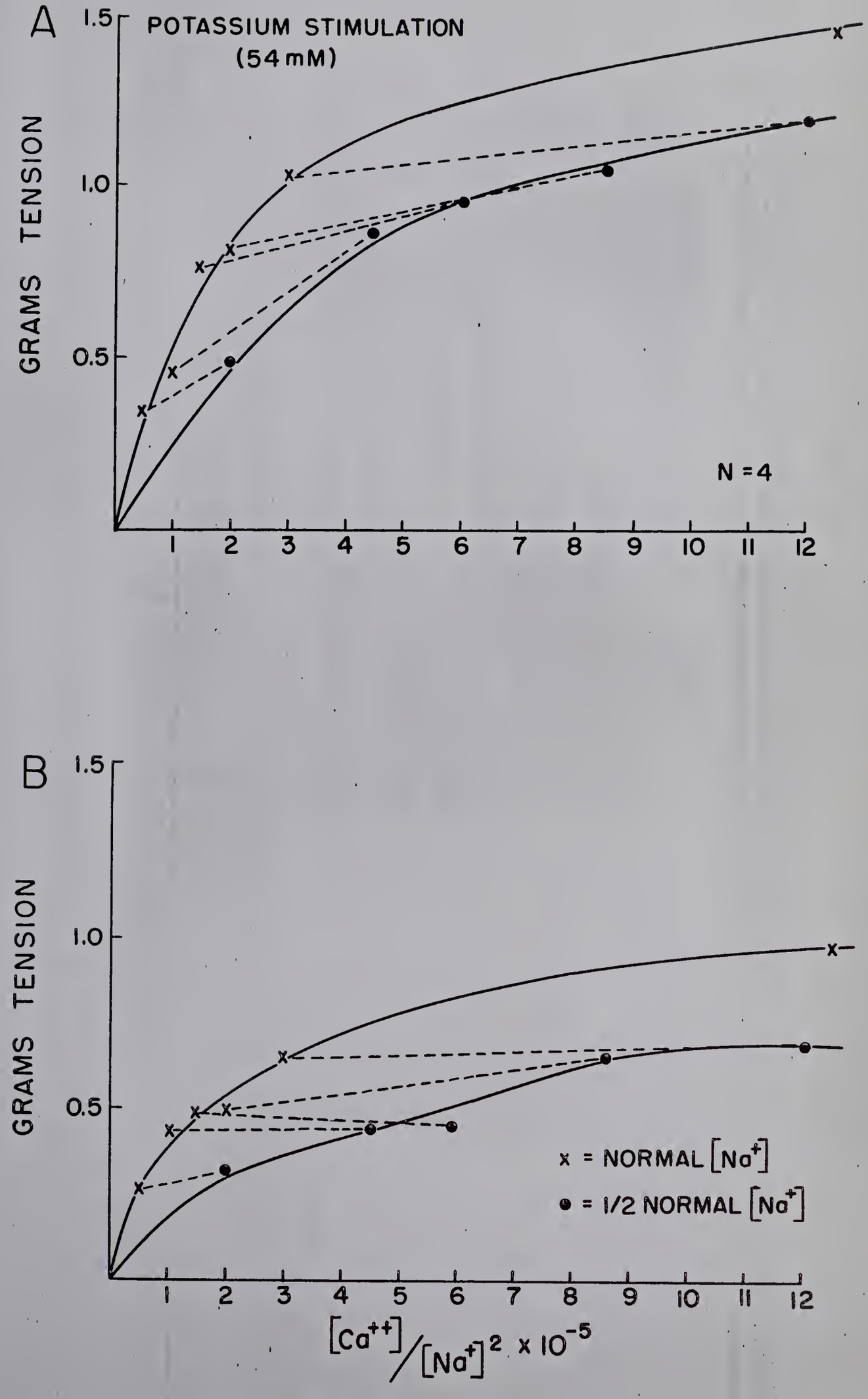

A
POTASSIUM STIMULATION
(54 mM)
GRAMS TENSION
1.5
1.0
0.5
N = 4
1 2 3 4 5 6 7 8 9 10 11 12
B
GRAMS TENSION
1.5
1.0
0.5
x = NORMAL [Na+]
● = 1/2 NORMAL [Na+]
1 2 3 4 5 6 7 8 9 10 11 12
[Ca++] / [Na+]2 x 10-5

Figure 10.

Effect of Foreign Anions on the Contractile Response of VSM to Potassium Sulfate.

The responses were obtained in normal Krebs bicarbonate solution (control) and in 'Anion' solutions containing Br, NO_3, I, and SCN as a substitute for chloride ions. The tissues were exposed for 1 minute and 15 minutes to foreign anions before stimulation with potassium sulfate. Each response is a mean of 4 to 8 tissues. The straight lines at the top of each response indicate the standard error. Changes in responses, expressed as percentage of control responses are tabulated below. The corresponding data are found in Appendix II, Table 6.

Percent Change in Response.

K_2SO_4 (mM)	8.		16.		32.		64.	
Exposure to Anion	1 Min.	15 Min.	1 Min.	15 Min.	1 Min.	15 Min.	1 Min.	15 Min.
Br	0	∞	24.8	61.6	5.3	7.7	6.8	6.0
NO_3	∞	∞	68.9	111.1	18.3	18.2	15.5	3.5
I	∞	∞	175.0	216.0	35.0	13.1	21.4	12.8
SCN	∞	∞	260.0	423.0	57.0	40.0	9.1	3.0

FIGURE 10

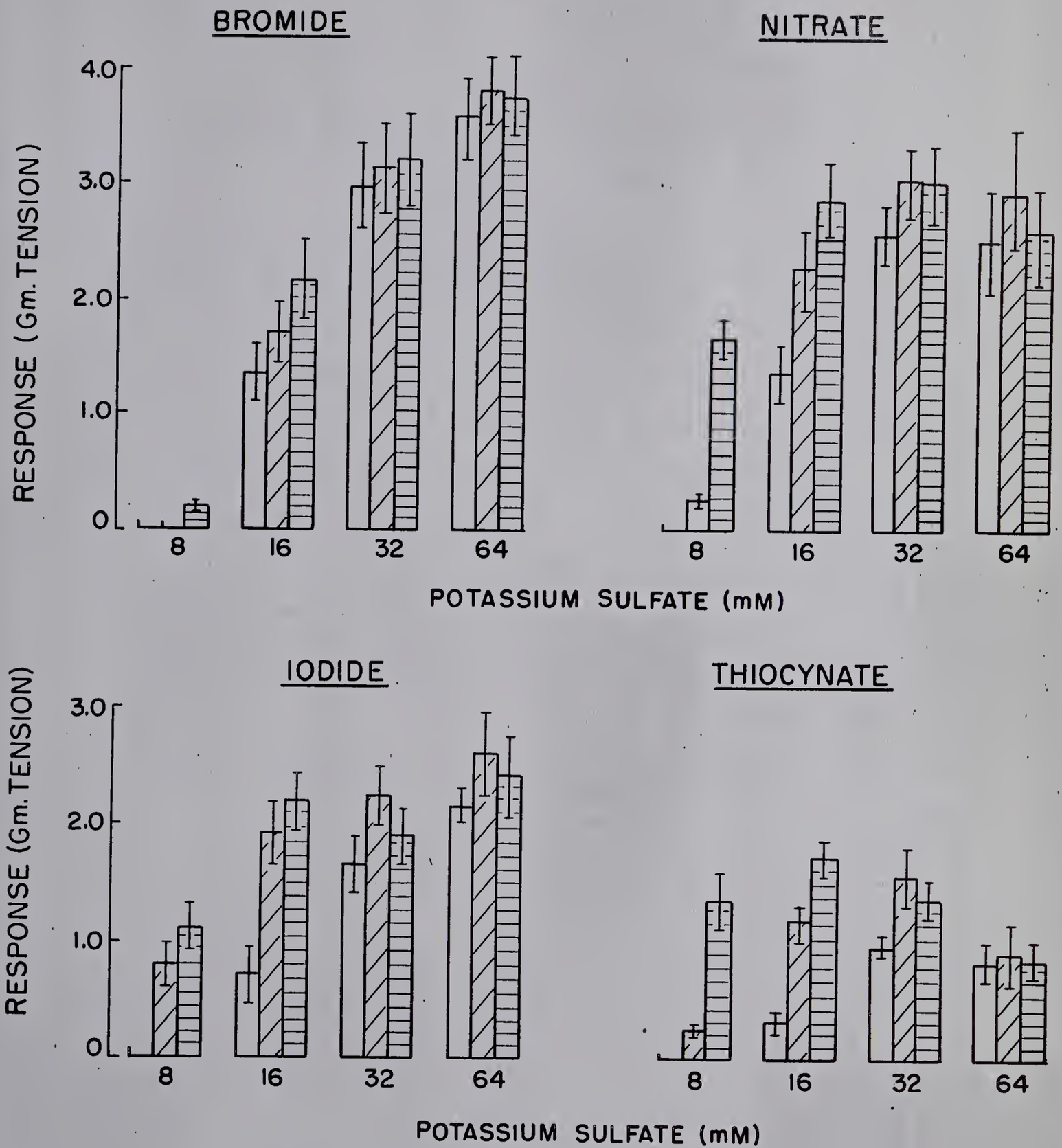

BROMIDE
NITRATE
IODIDE
THIOCYNATE
RESPONSE (Gm. TENSION)
4.0
3.0
2.0
1.0
0
8
16
32
64
POTASSIUM SULFATE (mM)
LEGEND
NORMAL KREBS - CONTROL
ANION SOLUTION - 1 MINUTE EXPOSURE
ANION SOLUTION - 15 MINUTE EXPOSURE

Figure 11.

Effect of Foreign Anions on the Contractile Response of VSM to Noradrenaline.

The responses were obtained in normal Krebs bicarbonate solution (control) and in 'Anion' solutions containing Br, NO_3, I, and SCN as a substitute for chloride ions. The tissues were exposed for 1 minute and 15 minutes to foreign anions before stimulation with noradrenaline. Each response is a mean of 4 to 8 tissues. The straight lines at the top of each response indicate the standard error. Changes in responses, expressed as percentage of control responses, are tabulated below. The corresponding data are found in Appendix II, Table 7.

Percent Change in Response.

NA (μg/ml)	0.1		0.2		0.4		1.0	
Exposure to Anion	1 Min.	15 Min.	1 Min.	15 Min.	1 Min.	15 Min.	1 Min.	15 Min.
Br	15.42	18.94	14.1	9.19	15.31	19.76	4.67	8.77
NO_3	12.9	42.9	2.4	20.6	1.17	22.4	12.3	20.0
I	56.33	42.89	50.68	46.57	46.51	36.68	42.98	14.61
SCN	82.05	29.23	64.4	3.27	56.54	4.21	58.33	13.54

Figures that are underlined indicate inhibition of response.

FIGURE II

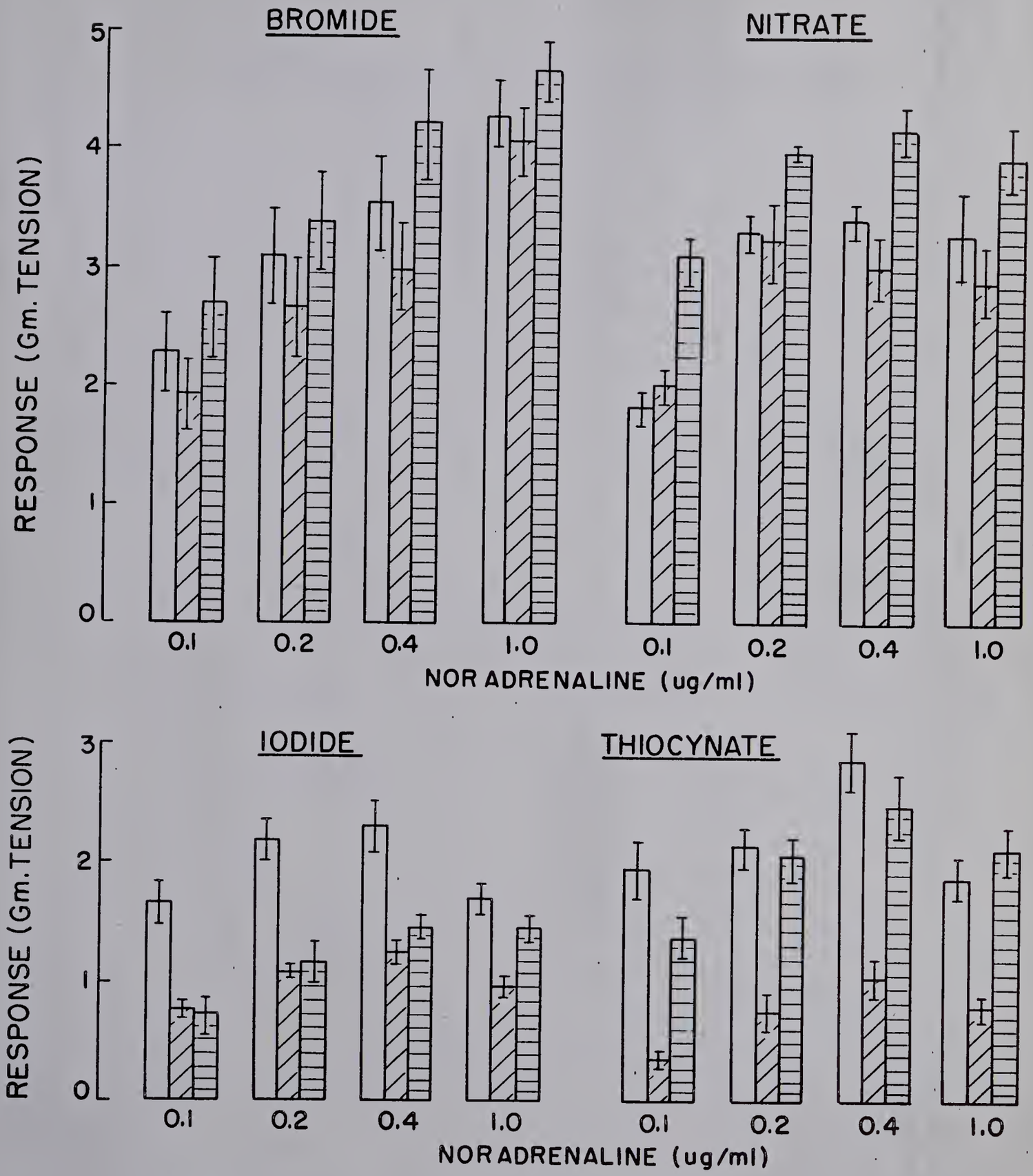

BROMIDE
NITRATE
RESPONSE (Gm. TENSION)
5
4
3
2
1
0
0.1
0.2
0.4
1.0
0.1
0.2
0.4
1.0
NOR ADRENALINE (ug/ml)
IODIDE
THIOCYNATE
RESPONSE (Gm. TENSION)
3
2
1
0
0.1
0.2
0.4
1.0
0.1
0.2
0.4
1.0
NORADRENALINE (ug/ml)
LEGEND
NORMAL KREBS - CONTROL
ANION SOLUTION - 1 MINUTE EXPOSURE
ANION SOLUTION - 15 MINUTE EXPOSURE

Figure 12.

Effect of Foreign Anions on the Contractile Response of VSM to Electrical Stimulation.

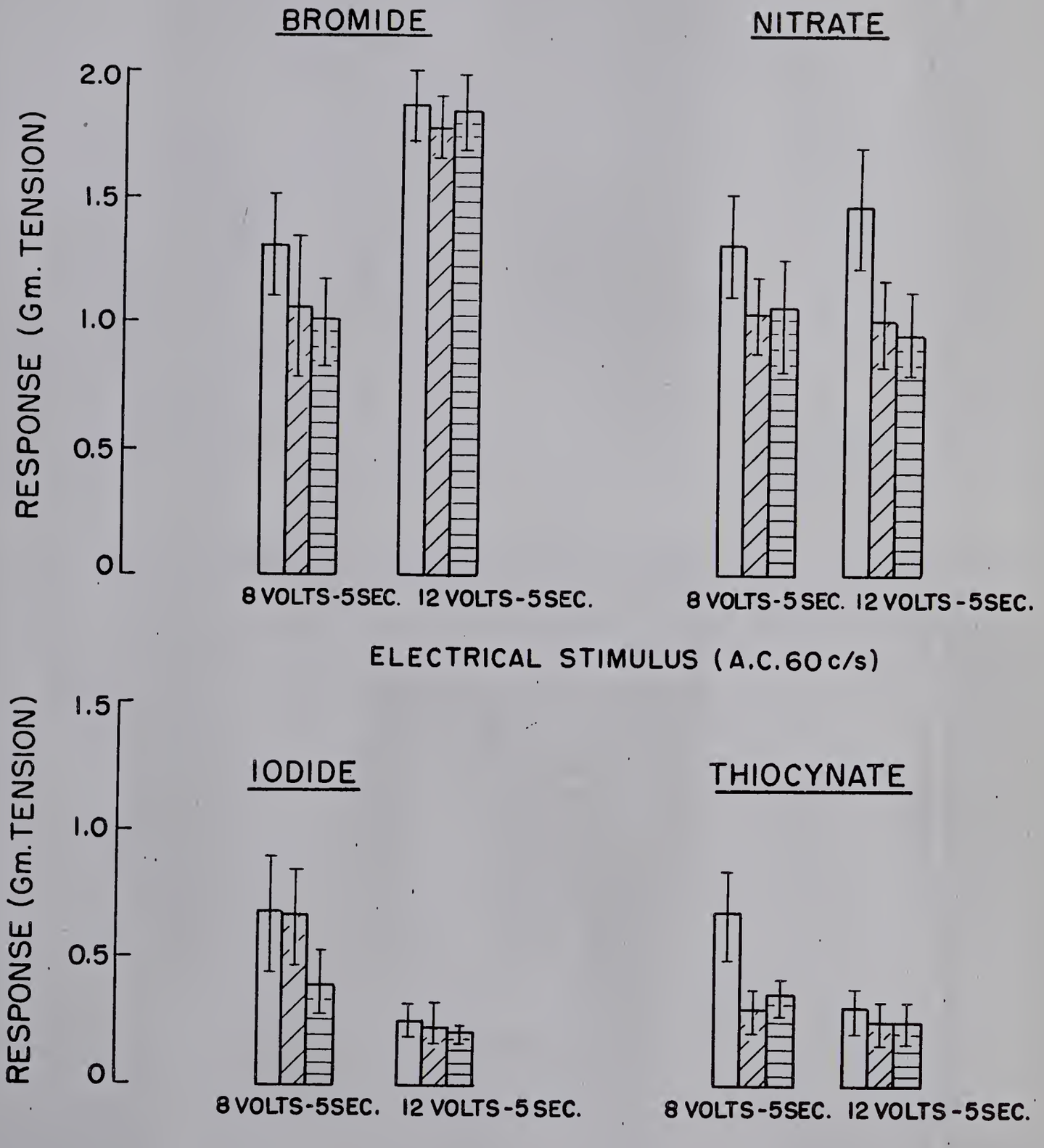
The responses were obtained in normal Krebs bicarbonate solution (control) and in 'Anion' solutions containing Br, NO_3, I, and SCN as a substitute for chloride ions. The tissues were exposed for 1 minute and 15 minutes to foreign anions before stimulation with electrical current. Each response is a mean of 4 tissues. The straight lines at the top of each response indicate the standard error. Changes in responses, expressed as percentage of control response, are tabulated below. The corresponding data are found in Appendix II, Table 8.

Percent Change in Response.

Voltage (a.c. 60c/s.)	8V-5Sec.		12V-5Sec.	
Exposure to Anion	1 Min.	15 Min.	1 Min.	15 Min.
Br	18.0	21.8	4.3	1.61
NO_3	22.5	19.4	30.0	33.6
I	0	48.0	4.20	1.25
SCN	54.6	48.5	16.1	16.1

Figures that are underlined indicate inhibition of response.

FIGURE 12



LEGEND

| | NORMAL KREBS - CONTROL

▨ ANION SOLUTION - 1 MINUTE EXPOSURE

▤ ANION SOLUTION - 15 MINUTE EXPOSURE

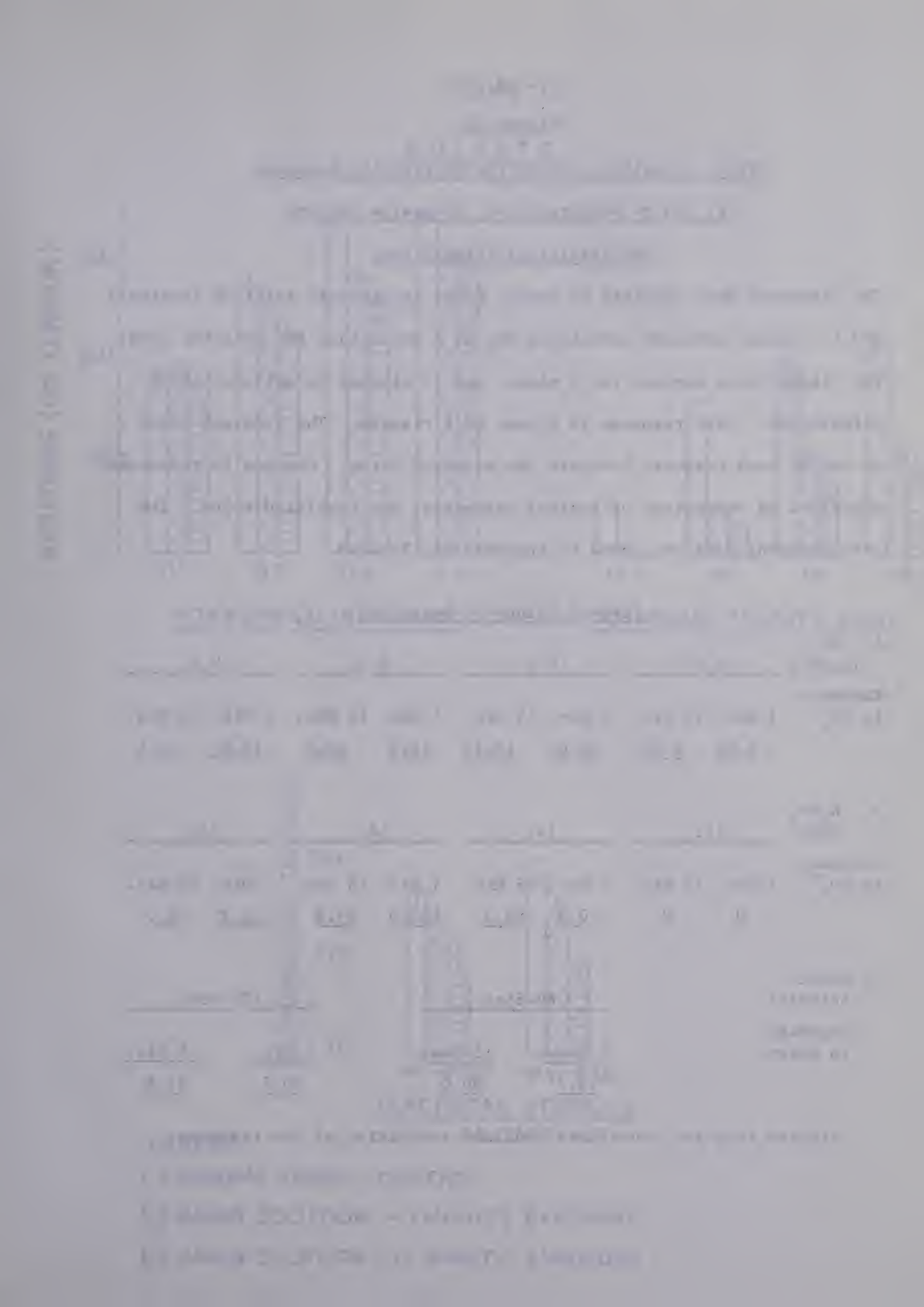


Figure 13.

Effect of Sulfate Ion on the Contractile Response of VSM to Noradrenaline, Potassium Sulfate and Electrical Stimulation.

The responses were obtained in normal Krebs bicarbonate solution (control) and in 'Anion' solution containing SO_4 as a substitute for chloride ions. The tissues were exposed for 1 minute and 15 minutes to sulfate before stimulation. Each response is a mean of 4 tissues. The straight lines at top of each response indicate the standard error. Changes in responses, expressed as percentage of control responses, are tabulated below. The corresponding data are found in Appendix II, Table 9.

Percent Change in Response.

A. NA (µg/ml)	0.05		0.1		0.2		0.4	
Exposure to SO_4^{--}	1 Min.	15 Min.	1 Min.	15 Min.	1 Min.	15 Min.	1 Min.	15 Min.
	8.62	2.87	10.63	18.13	18.8	26.5	11.67	16.1

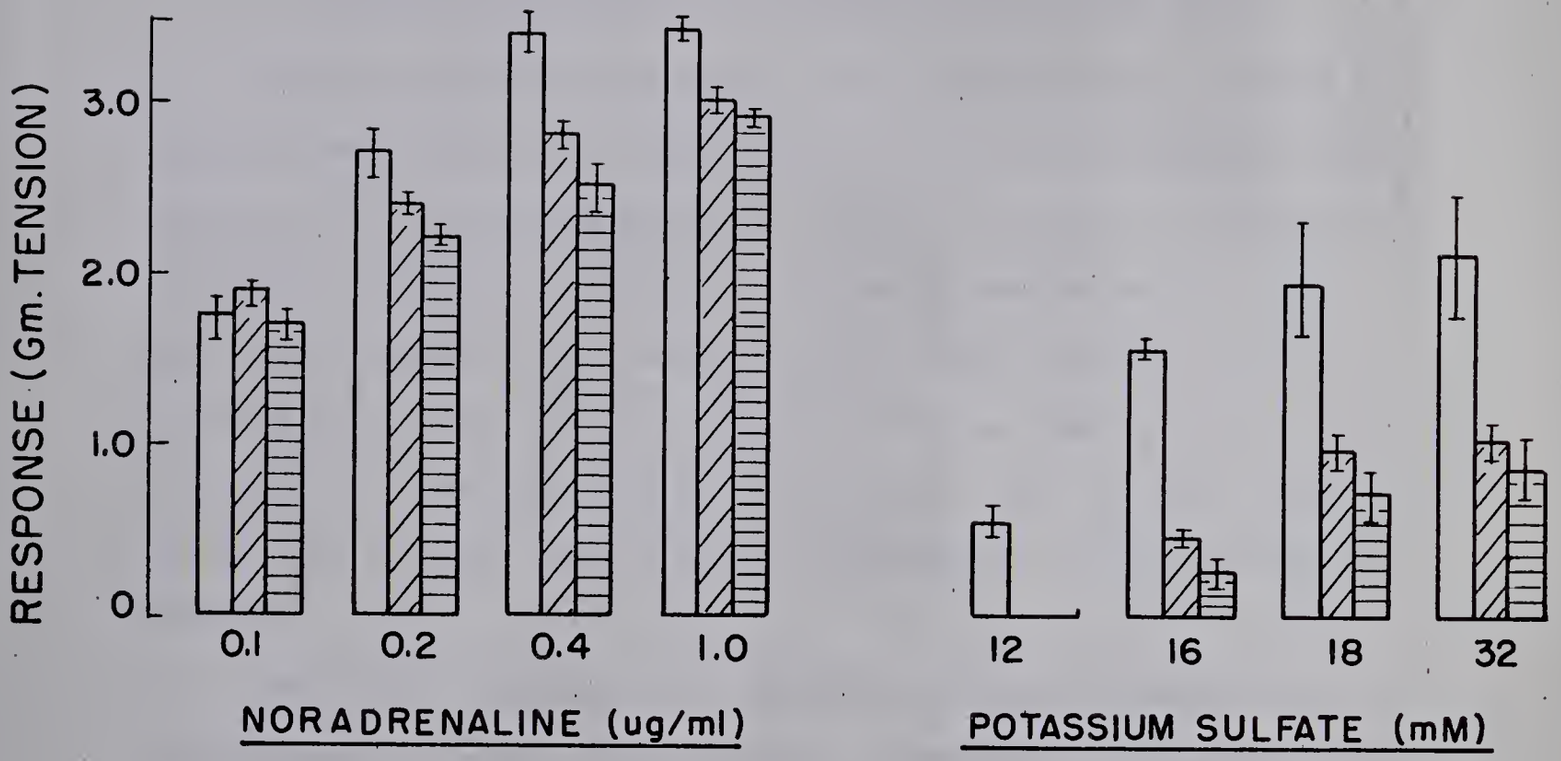
B. K_2SO_4 (µM)	12.		16.		18.		32.	
Exposure to SO_4^{--}	1 Min.	15 Min.	1 Min.	15 Min.	1 Min.	15 Min.	1 Min.	15 Min.
	0	0	70.3	84.5	52.0	64.8	51.2	59.0

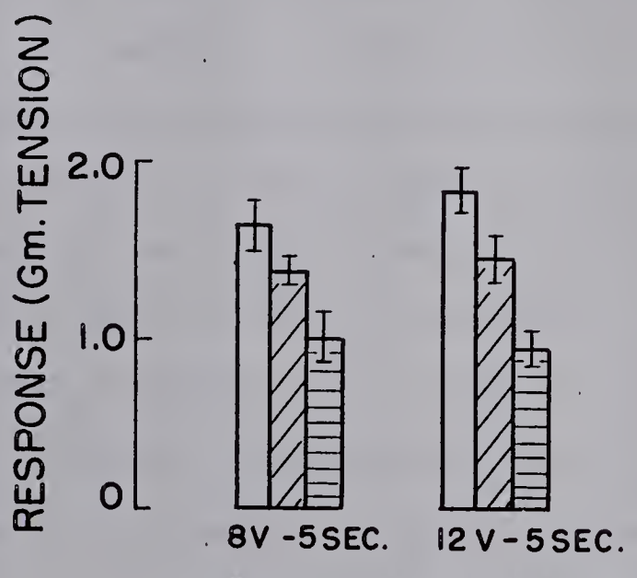
C. Elect. Stimulus	8V-5Sec.		12V-5Sec.	
Exposure to Anion	1 Min.	15 Min.	1 Min.	15 Min.
	16.7	40.0	20.7	41.8

Figures that are underlined indicate inhibition of the response.

FIGURE 13

SULFATE





LEGEND

| | NORMAL KREBS - CONTROL

/// ANION SOLUTION - 1 MINUTE EXPOSURE

≡ ANION SOLUTION - 15 MINUTE EXPOSURE

DISCUSSION

A. Effects of Changes in CO_2 and Calcium Concentration.

The mechanism by which carbon dioxide depresses the response of vascular smooth muscle to noradrenaline has not been definitely determined but it has been suggested that the site of action is neither the adrenergic receptor nor the final contractile mechanism (45). It therefore appeared to be somewhere between these extremes in the stimulus contraction coupling process and may be associated with the availability of calcium ions for contraction; that is, with either the release of bound calcium or the permeation of calcium outside the membrane.

Bohr (7, 8) indicated that adrenaline induced contractions of aortic muscle consist of slow and fast components. He suggested that the magnitude of the fast component of contraction is an indication of the membrane excitability. The slow component on the other hand may be an indication of the degree of excitation-contraction coupling. Bohr found that the slow component of contraction failed first during calcium depletion. Daniel (13) has suggested that the fast phase of the contraction may be caused by calcium release from sequestering sites in the tissues and some calcium entrance from interstitial fluid following stimulation, while the slow phase of contraction may result from a slow continued entrance of calcium from interstitial fluid.

In these experiments a satisfactory analysis of the two phase response induced by noradrenaline could not be made owing to the

difficulty in distinguishing the point of flexion where the fast phase ended and the slow phase began. The response of rabbit aorta tissues to electrical stimulation also produces a biphasic response in which the distinction between the slow and fast phases is much sharper than that in the drug induced response. Furchgott (25) showed that after treatment of rabbit aorta tissues with dibenamine, an α-receptor adrenergic blocking agent, electrical stimulation produced only the fast phase. Yates and Gillis (60) tested the responses of tissues from reserpine treated animals, depleted of noradrenaline, and found that the slow phase was abolished. From these observations the authors concluded that the slow phase of contraction, after current flow has ceased, may be due to a liberation of an adrenaline like substance in the artery during electrical stimulation.

In this investigation it was found that both high CO_2 and low calcium concentration reduced the slow phase of contraction. This observation brought up the question of whether dibenamine, reserpine and high CO_2 had their depressive effects through a common mechanism such as a decrease in the availability of calcium ions. Further observation showed that INA like high CO_2 also abolished completely and reversibly the slow phase of contraction. If INA also in some way interferes with the availability of calcium, then its action is preceded by a combination with the β-adrenergic receptor sites. In tissues treated with DCI, a β-receptor adrenergic blocking agent, INA failed to depress the slow phase of the response, but under similar conditions high CO_2 still caused a depression of the slow phase. This result

supports the earlier view of Nash et al. (45) that the depression caused by CO_2 is not exerted through interaction with β-receptor sites, but is perhaps intimately related to availability of calcium to the contractile machinery.

The mechanisms by which the availability of calcium ions could be impaired by high CO_2 is not known. If a sodium calcium competition, analogous to that in the frog heart muscle (43), also exists in VSM then it is possible that CO_2 may be acting by reducing the formation of an active calcium compound (CaR). Alternately it may promote the formation of an inactive sodium compound (NaR). A further possibility exists that a high concentration of CO_2 may interfere with the permeation of calcium by reducing the binding capacity of calcium sites, the permeability of cell membranes, or active transport mechanisms in the cell membranes.

B. Sodium and Calcium Competition in VSM.

Dodd and Daniel (15) showed that a considerable quantity of sodium is bound in the rabbit aorta tissue, and Headings, Rondell, and Bohr (30) have indicated that a mucopolysaccharide present in the medial layer of the arteries, possibly in the cell membrane is a probable site for binding of the cations. Sodium bound to this polyanion appears to be readily exchangeable with hydrogen and potassium ions. It is possible that sodium and calcium ions compete for anionic binding sites on such a tissue constituent, to form either a calcium compound activating contraction, or a sodium compound which is inactive. If

such a sodium-calcium competition exists in VSM, then it is possible to explain the potentiation and inhibition of the contractile response of the muscle in terms of relatively greater formation of the active calcium compound, or the inactive sodium compound respectively. Some workers (5, 58) who have reported a potentiation of the contractile response in low sodium solution have attributed this effect to a sodium-calcium competition in this muscle.

The data from experiments in this study confirm the findings that calcium is essential for the contraction of VSM of rabbit aorta when the latter is stimulated electrically, by noradrenaline or by potassium, but they also indicate that in this tissue the contractile process is not controlled by a competition between sodium and calcium. If a simple competition between sodium and calcium for a specific anion existed and controlled the availability of calcium for the contractile process, we should reasonably expect only one response value for a single value of the activity ratio $[Ca^{2+}] : [Na^{+}]^2$, as in the case of frog heart muscle (43). Thus all the responses to the same stimulus, at different values of the activity ratio, should be scattered along a common regression line (43). In these experiments any single value of the activity ratio $[Ca^{2+}] : [Na^{+}]^2$ showed two distinct responses, their size depending upon $[Ca^{2+}]$, thus reducing the probability of a competition between sodium and calcium based on the hypothesis of Lüttgau and Niedergerke (43). A similar lack of sodium and calcium competition in the uterine smooth muscle has been reported by Daniel and coworkers (14). These results serve to emphasize that in this regard smooth muscle differs from the

cardiac muscle.

The frequently observed changes in the response associated with the changes in $[Na^+]$ are apparently unrelated to $[Ca^{2+}]$ of the medium, but may be due to an imbalance of the normal ratio of $[Na^+]_i : [Na^+]_o$ in the smooth muscle cells. It might be expected on the basis of this hypothesis that effects of changes in the external sodium would be most prominent within a very brief period after such changes, and would be reduced as the internal sodium reached a new equilibrium. Such a hypothesis would fit the present data, for a brief exposure to a sodium deficient solution enhanced the contractile response, whereas after a prolonged exposure there was a little change in the response. That this change is probably not directly associated with the contractile mechanism per se.is indicated by the observation that the maximum response was not affected. The observed potentiation disappeared as the intensity of the stimulation was increased, that is, when the dose of noradrenaline was increased. The mechanism by which this elevated ratio of $[Na^+]_i : [Na^+]_o$ potentiates the response has not been determined, but it may be that it is due to a temporary instability of the membrane which allows, in response to a stimulus, a more effective intracellular release of calcium and/or a passage of calcium through the cell membrane. In addition there is a possibility that the increased sensitivity is due to an imbalance in the anion concentrations similar to that reported by Hodgkin and Horowicz (34). Single muscle fibres were shown to increase their twitch response to a constant stimulation when the bath medium contained a larger, more slowly

permeating anion (SCN, NO_3, I or Br) substituted for the chloride ion. In these experiments an anion imbalance is very likely to have occurred because sucrose was used to displace sodium chloride.

Friedman et al. (24) have observed the activating and the potentiating effects of reducing the external sodium concentration in both rat colon and VSM, and have proposed that a sodium gradient across the cell membranes may be a primary determinant in the muscle tone and the control of blood pressure. These data appear to be in agreement with their findings, and the fact that the potentiating effect is short lived suggests that the postulated high $[Na^+]_i$: $[Na^+]_o$ ratio is also transient, and that the normal ratio is soon established. Whether the sodium ratio or its effect on calcium or on another ion in the membrane is the primary determinant of the muscle contractility remains to be determined. In all smooth muscles that to date have been adequately studied (12, 14, 27) sodium fluxes across the membrane have been found to be very fast, so that a rapid restoration of the sodium gradient is to be expected. This restoration of the local sodium gradient in the region of the membrane could be rapidly accompanied by a shift of a relatively small amount of sodium into the binding sites. A rapid uptake of sodium into the membrane binding sites unoccupied by calcium implies an exchange of sodium for some other cation such as potassium or hydrogen and that the $[Na^+]_o$ may influence the $[Na^+]_i$ by initiating a shift in the bound cations throughout the membrane. The presence of a system for rapidly reestablishing the normal sodium gradient and the excitability of the cell makes it difficult to associate an altered

sodium concentration gradient with a prolonged condition such as the chronic hypertension. It is possible, however, that alterations in the number of locations of the binding sites, including interstitial ones, may lead to structural changes in the muscle cells as well as to an altered excitability and contractility associated with hypertension.

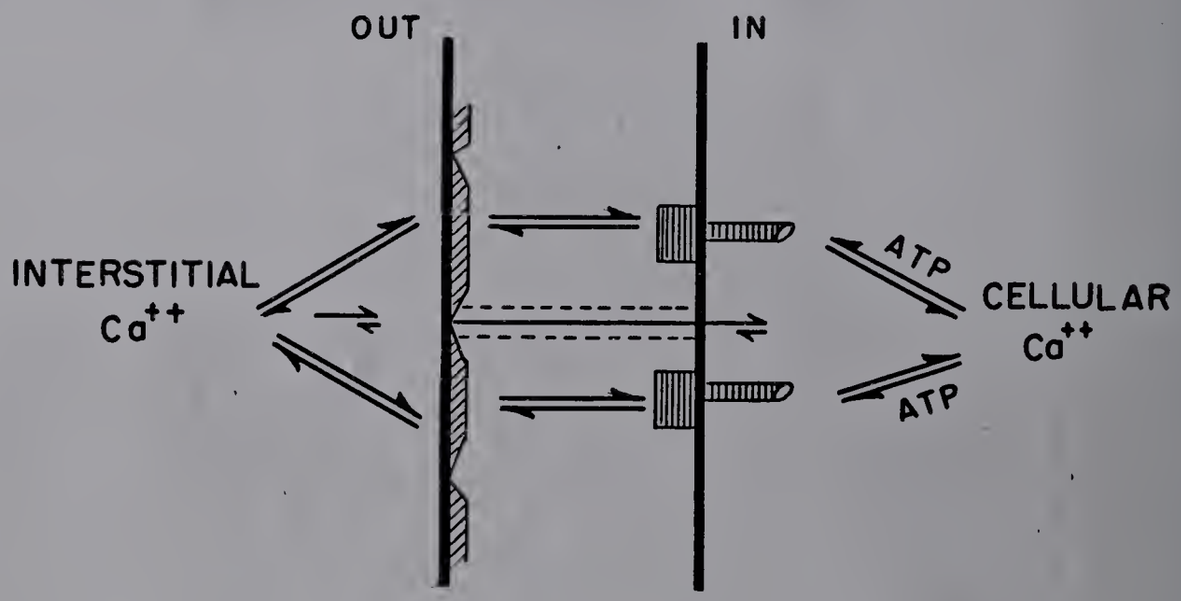
C. Effect of Inorganic Anions on Contractility of VSM.

When chloride ion in the normal Krebs bicarbonate solution bathing the tissues is substituted with foreign anions of varying membrane permeability, the overall effect on the contractile response of the muscle may be due to an individual effect on any of the three stages in muscular contraction; 'Membrane Phenomena', 'E-C Coupling', 'Protein Contraction', or it may be a sum total of their action on all the stages. The evidence from other muscles especially the skeletal muscle shows that the effect of foreign anions on the contractility of this muscle is related to an entry of calcium ions into the muscle cells (3, 21). This entry of calcium represents at least one step of the excitation-contraction coupling process. The evidence for a similar effect of anions on the vascular smooth muscle is incomplete. It has been suggested from the work on mesenteric arterial segments from the intestine that the replacement of chloride by various organic and inorganic anions potentiates the response induced by l-adrenaline, and that such increase in contractility is the result of a greater calcium diffusion across the cell membrane (57). The experiments in this investigation show that the contractile response of the rabbit aorta tissue in the presence of foreign anions

FIGURE 14

A MODEL OF CALCIUM EXCHANGE ACROSS THE CELL MEMBRANE

A SERIES – PARALLEL MODEL



Superficial Ca^{2+} binding Site

Sequestered Ca^{2+} binding Site

Pores for Ca^{++} diffusion available when superficial Calcium removed

varies with the type of the anion, the nature of stimulating agent, the dose of stimulating agent, and the time of exposure to a particular anion before the stimulus is applied.

It has been suggested by Hinke and Wilson (32) that potassium sulfate and noradrenaline, the agents employed in these experiments, produce a contraction of VSM by utilising two fractions of the membrane calcium. They proposed that high potassium utilizes loosely bound calcium which is dependent on the external calcium, and the permeability state of the membrane. On depolarisation this calcium is easily mobilised. Noradrenaline on the other hand is thought to produce contraction primarily by the release of more tightly bound calcium fraction, which has a limited dependence on the permeability state of the membrane and the external calcium. However, through a secondary depolarising action noradrenaline may also cause a release of the loosely bound calcium. Daniel (13) suggests that loosely bound or superficial calcium is located on the outer side of the cell membrane while the tightly bound or sequestered calcium is located on the inner side of the cell membrane. These two binding sites of calcium may be in equilibrium.

The concept of two sites of calcium binding is illustrated by a 'Parallel-Series Model' put forward by Daniel (13). This model allows an interpretation of the differences of contractile effects produced by foreign anions, when potassium or drug stimulus are used, in terms of calcium permeation into the myoplasm from these two sites. A potentiation of the contractility produced by Br, NO_3, I and SCN in response to

potassium stimulation of VSM of rabbit aorta may be attributed to an increase in the availability of superficial calcium brought about by an increased binding of the external calcium to the membrane and increased permeation through the membrane in the presence of these anions. When noradrenaline is used to produce contraction, in the presence of same anions, the varying effect could be explained in terms of increase in the binding and rebinding of calcium at the intracellular sequestered calcium binding sites. The foreign anions Br, NO_3, I, and SCN may increase the quantity and the firmness of calcium binding at the sequestered site as a result of increase in calcium binding at the superficial sites. If the intracellular calcium is more firmly bound in the presence of anions, a stronger stimulus may be required to release it by noradrenaline. In the presence of some foreign anions the response to noradrenaline would be depressed owing to the firm binding and difficult release of sequestered calcium. Furthermore, this inhibitory effect would be reduced, as the dose of the drug was increased, thus allowing a greater release of the bound calcium into the myoplasm. In these experiments it was found that anions such as I and SCN , which are believed to bind calcium more firmly than other anions such as Br and NO_3, do indeed inhibit the response of rabbit aorta tissues induced by noradrenaline. This inhibition was found to be greater at low dose levels of noradrenaline than that observed at higher doses. It is also possible to account for the inhibition of response produced in the presence of I and SCN, and potentiation in presence of Br and NO_3, in terms of the rebinding of intracellular

calcium once it has been released by the drug stimulus. Both Br and NO_3 are less permeable than chloride through the cell membrane but they are more permeable than I and SCN . Thus in a given exposure time, Br and NO_3 may reach a sufficiently high concentration inside the cell to pair with calcium and transport the latter to the myoplasm at a faster rate. However, I and SCN owing to their relatively low permeability through the cell membrane may not be able to attain a sufficiently high concentration in the same interval of time. Consequently the calcium released, being unable to pair with the anion, may be rebound to the site from which it was released by the drug. The results of experiments using I and SCN support this view, in that as the exposure time to I and SCN was increased from 1 minute to 15 minutes the degree of inhibition became less at all dose levels of the drug. The effect of anions on the binding of sequestered calcium in the smooth muscle must remain speculative because such an effect on the intracellular calcium binding sites has not been shown even in the skeletal muscle.

In addition to the primary non depolarising action through a release of sequestered calcium, noradrenaline also has a secondary depolarising action which is exerted by mobilisation of superficial calcium. The greater potency of Br and NO_3 , and the lower potency of I and SCN than the chloride ion, in response induced by noradrenaline at a single dose level, may be the result of a balance between the influence of foreign anions on the mobilisation of superficial and the sequestered calcium.

The mechanism by which the anions bring about increased binding of the membrane calcium and an increased permeation of this calcium has not been determined. It is possible that the anions through their adsorption on the membrane sites that bind calcium may alter the nature or the distribution of these sites causing a saturation of these sites with external calcium. When the membrane is depolarised, the calcium in the membrane may associate with the available anions (by a depression of the 'dissociation field effect'--see below) and diffuse as ion pairs into the myoplasm. These ion pairs may subsequently dissociate and release the calcium ions. From this standpoint the calcium content of the membrane provides an adequate source of calcium that will ionise on entering the fibre. The rate of entry of calcium or the quantity of the ionised calcium entering the myoplasm may be expected to be a factor in delaying the rebinding of calcium.

The experiments in this investigation showed that the order of contractile activity in VSM produced in the presence of anions and in response to potassium stimulation was: $SCN > I > NO_3 > Br > Cl$. Differences in the size of contractile responses in presence of anions under similar conditions of stimulation, can be attributed to the ease with which calcium can pair with these anions and diffuse through the membrane. The ion pair hypothesis of Shanes (51) discussed below provides the basis for the order of contractile activity stated above, which is the same as the order of increase in the polarisability of these anions: $SCN > I > NO_3 > Br > Cl$.

The ion pair formation is basically an association of two or more

oppositely charged ions by virtue of electrical attraction and/or other forces in a region of low effective dielectric constant. Some of the conclusions from studies on ion pair formation are pertinent to this discussion.

a. Below a critical dielectric constant depending on the effective radii and on the polarisability of the ions, ion association occurs.

b. This association is greater, the smaller the dielectric constant, the smaller the effective radii, and the greater the polarisability of the components in the system.

c. The greater the ion concentration, the greater is the association, the quantitative relationship being given by association constants, of which there may be several if the multiple ion pairs form.

In the transition from the molecular to the ionised form there are three stages:

$$\text{Molecule} \rightleftharpoons \text{Ion Pair} \rightleftharpoons \text{Cation} + \text{Anion}$$

The effect of a high electrical field strength, e.g., the field associated with cell membrane, is to shift the above equilibrium to the right. This is termed the 'dissociation field effect'. It has been estimated that across a membrane of 100 Å thickness the field effect may be equivalent to a field of 100,000 Volts/cm. On depolarisation with a drug or potassium, ion pairs form more readily due to the removal of the dissociation effect.

Experiments in which electrical stimulation was used to observe the effects of anions on the biphasic response induced by this method showed that the total response was inhibited in the presence of all anions,

regardless of the strength of the stimulus, or the time of exposure to the anion. The recovery of the control responses in the normal Krebs solution was subsequently impaired. A satisfactory explanation to account for these effects is not possible without a knowledge of the effects of an A.C. voltage on the electrical properties of the solution.

In a solution where sodium chloride was replaced with an equimolar amount of sodium sulfate, the contractile response induced by potassium, noradrenaline, and electrical stimulation was depressed in all cases. However, the responses evoked by potassium were inhibited much more than those induced by noradrenaline. Unlike the anions Br^-, NO_3^-, I^- and SCN^-, sulfate does not permeate through the cell membrane. It forms a complex with calcium ions making the latter unavailable for binding to the membrane sites. Thus in the presence of sulfate the superficial calcium sites, which depend on external calcium far more than sequestered calcium sites, may not be fully saturated with calcium. The contractions produced by potassium, which is thought to mobilise superficial calcium, were consequently depressed in external medium containing sulfate ions. In the presence of low external calcium the sequestered calcium binding sites could still be saturated with calcium. Noradrenaline, which acts primarily by mobilisation of sequestered calcium, produced contractions which were depressed compared to the control response but were far greater than those induced by potassium. The results from these experiments support the concept of two stores of calcium and their utilisation by various stimulating agents.

SUMMARY AND CONCLUSIONS.

1. Electrical stimulation of isolated rabbit aorta tissues produced a dual response consisting of a fast initial contraction and a slow delayed rise in tension. The latter shows a greater dependence on the external calcium than does the initial response. On lowering the calcium content of the external medium the delayed response is reduced more than the initial response, and on increasing the external calcium the delayed response increases in size more than the initial response. High CO_2 and isoproterenol also depress the delayed response more than the initial response. Dichloroisoproterenol prevents the depressive effect of isoproterenol on the delayed response, but not that of high CO_2. It is suggested that the depression of contractility caused by high CO_2 is not mediated through adrenergic receptor sites but is related to an interference with the availability of calcium ions for contraction.

2. Calcium ions are essential for contractile response of vascular smooth muscle induced electrically and by noradrenaline. Maximum responses are elicited in the presence of 0.60 to 1.25 mM of calcium in the Krebs bicarbonate solution. Lowering the sodium concentration in the external medium potentiates the response induced by low doses of noradrenaline but not that induced by high doses. Tests for the presence of a sodium-calcium antagonism show that such a competition does not exist in the vascular smooth muscle, and the potentiation of response observed in low sodium can not therefore be explained on the basis of this competition. The results have been interpreted to relate low sodium

potentiation to a reduction of $[Na^+]_o$: $[Na^+]_i$ gradient.

3. Substitution of chloride ions in normal Krebs bicarbonate solution with foreign anions, Br, NO_3, I, and SCN, causes potentiation of responses induced by potassium. The potentiation increases when exposure to anion is increased from 1 minute to 15 minutes but it decreases as higher concentrations of potassium are used to produce the tissue responses. In the case of responses induced by noradrenaline, Br and NO_3 potentiate the contractions but I and SCN depress them. The responses increase in size on prolongation of exposure to anions from 1 minute to 15 minutes. In the case of I and SCN the response is still depressed after a 15 minute exposure. When electrical stimulus is used under similar conditions all of the above anions produce an irreversible depression of contractile response.

4. Substitution of chloride with sulfate results in an inhibition of response induced by potassium, noradrenaline and electrical stimulation. This inhibition is observed regardless of the stimulus and the exposure to sulfate ion. The degree of inhibition is greater in the case of responses evoked by potassium than those induced by noradrenaline. The sulfate ion through complexation with calcium reduces the effective concentration of external calcium.

5. It is concluded that potassium stimulates contraction by mobilisation of calcium from superficial calcium binding sites located on the outer side of the cell membrane, whereas noradrenaline stimulates contractions primarily by liberation of calcium from intracellular sequestered calcium sites. With the exception of sulfate, foreign

anions appear to increase the binding of calcium at these sites, and also increase its permeation into the myoplasm through the formation of readily diffusible ion pairs. The contractions induced by potassium in the presence of foreign anions are potentiated as a result of increase in calcium binding at superficial sites and an increase in its permeation through the membrane in ion pair form. The responses induced by noradrenaline under similar conditions are depressed perhaps due to a reduction in the release of calcium ions from the sequestered sites. This reduction may be due to an increase in the immediate rebinding of calcium after it has been released.

6. This work indicates that in vascular smooth muscle the availability of calcium for contraction can be altered through changes in the calcium content and the anionic content of the tissue bath medium. Although it appears that a high CO_2 concentration depresses the contractility through a reduction in the calcium availability it is still not clear how this proposed reduction takes place and what sites of calcium are affected by high CO_2. The answer to these problems may be found in further experiments on the effect of high CO_2 on the responses of tissues in a medium of varied calcium content. As it has been indicated that stimulating agents such as potassium and noradrenaline mobilize calcium from separate stores of membrane calcium, these methods of stimulation may help to localize the site of action of carbon dioxide.

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APPENDIX I

Preparation of Solutions.

All solutions used to bathe the tissues were prepared in distilled water which had been passed through a deionizer.

Normal Concentrated Salt Solutions:

Sodium Chloride (NaCl)	82.60 grams
Potassium Chloride (KCl)	4.22 "
Calcium Chloride ($CaCl_2$)	3.36 "
Potassium Phosphate (KH_2PO_4)	1.94 "
Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	3.50 "

This mixture of salts is dissolved in water to produce a total volume of one litre.

Normal Krebs bicarbonate solution is prepared by mixing 100 ml. of the concentrated solution, 900 ml. of distilled water, 2 grams of glucose and 5 grams of sodium bicarbonate contained in 192 ml. of distilled water. The pH of this solution is 7.6. When aerated with 5% CO_2 and 95% O_2 mixture the pH is 7.35 to 7.40. This solution contains the following millimolar concentrations:

NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4.7H_2O$ 1.2 mM, $NaHCO_3$ 25 mM, glucose 10 mM.

Variation of Calcium.

The amount of $CaCl_2$ added to make concentrated solution is adjusted to required fraction of normal calcium. The osmolarity is maintained by adding equivalent amount of sucrose to the solutions.

Variation of Sodium.

The amount of NaCl added to concentrated salt solution is reduced by 71.5 mM. Equivalent amount of sucrose is substituted for sodium chloride.

The Krebs solutions containing adjusted concentrations of calcium and sodium are prepared in the same way as the normal Krebs bicarbonate solution.

Variation of Anion (Chloride).

Solutions in which chloride ion is substituted with a foreign anion are prepared by replacing sodium chloride in the normal Krebs solution with the sodium salt of an appropriate foreign anion. The solution containing bromide ions is prepared as:

NaBr	12.20 grams
KCl	0.35 "
$CaCl_2$	0.28 "
KH_2PO_4	0.16 "
$MgSO_4 \cdot 7H_2O$	0.30 "
Glucose	0.90 "
$NaHCO_3$	2.1 "

The salts are mixed and the solution is made up to one litre in distilled water. The millimolar concentration and the pH of this

solution is the same as that of normal Krebs solution. Solutions containing other anions are prepared by using the appropriate sodium salt in place of sodium bromide. The amounts used are:

$NaNO_3$	10.03 grams
NaI	17.70 "
NaSCN	9.56 "
Na_2SO_4	11.20 "

All anion solutions are prepared freshly before use.

APPENDIX II

Abbreviations.

Dose NA = Noradrenaline Dose (μg/ml.).

Gm. T. = Mean response of tissues in grams tension.

S.E. = Standard Error.

P = Probability (Students Paired t-test)

Each set of data in each table was obtained from a separate experiment or a group of experiments.

TABLE 1.

Influence of Calcium on the Dual Response of VSM to Electrical Stimulation.

External Calcium (mM)	Initial Response (Gm. T.)	Delayed Response (Gm. T.)
2.50	1.2	1.7
0.	0.55	0.30
0.08	0.70	0.88
0.16	0.70	0.87
0.33	0.78	1.05
0.66	0.90	1.30
1.20	0.90	1.15
2.50	0.85	1.10

TABLE 2.

Influence of Calcium on Response of VSM to NA and Electrical Stimulation.

Stimulus	Noradrenaline				Electrical Stimulation			
	0.1 µg/ml.		0.5 µg/ml.		8 Volts/Sec.		12 Volts/Sec.	
Fraction of Normal [Ca^{++}]	Gm. T.	% Response*	Gm. T.	% Response*	Gm. T.	% Response**	Gm. T.	% Response**
NORMAL (2.5 mM)	2.18±0.17	-	3.45±0.25	100	2.0 ±0.07	-	2.34±0.37	100
Ca-FREE + E.D.T.A.	0.13±0.02	3.5	0.35±0.04	10	0.39±0.06	17	0.66±0.14	28
1/32	1.03±0.13	29	2.1 ±0.40	61	0.69±0.12	29	1.15±0.18	49
1/16	0.94±0.24	27	2.44±0.38	68	0.59±0.07	25	1.10±0.18	47
1/8	1.44±0.33	41	2.88±0.30	85	0.96±0.13	41	1.44±0.22	62
1/4	1.59±0.28	45	3.1 ±0.33	90	1.11±0.19	47	1.71±0.27	73
1/2	1.84±0.10	53	2.99±0.32	87	1.39±0.19	59	1.73±0.29	74
NORMAL	1.79±0.31	52	2.97±0.35	86	1.03±0.22	44	1.55	66

* % Response to 0.5 µg/ml in Normal Calcium.

** % Response to 12 Volts-5 Sec. in Normal Calcium.

TABLE 3.

Tests for Sodium-Calcium Competition in VSM Using Noradrenaline and Electrical Stimulation.

Fraction of Normal [Ca] & [Na]		Ratio $[Ca^{2+}]:[Na^{+}]^2$	Response (Gm. T.) after exposure to reduced Na for 18 Minutes	Response (Gm. T.) after exposure to reduced Na for 1 Minute
A.				
1/6	1	0.75×10^{-5}	0.235 ± 0.04	0.90 ± 0.09
1/16	1/2	3.00×10^{-5}	0.240 ± 0.05	1.15 ± 0.10
1/12	1	1.125×10^{-5}	0.42 ± 0.08	1.10 ± 0.10
1/12	1/2	4.50×10^{-5}	0.62 ± 0.01	1.30 ± 0.12
1/8	1	1.5×10^{-5}	0.81 ± 0.19	1.40 ± 0.21
1/8	1/2	6×10^{-5}	0.91 ± 0.16	1.95 ± 0.27
1/16	1	2.124×10^{-5}	1.095 ± 0.21	1.55 ± 0.25
1/6	1/2	8.5×10^{-5}	0.96 ± 0.21	2.30 ± 0.31
B.				
1/32	1	0.375×10^{-5}	0.20 ± 0.02	0.104± 0.001
1/32	1/2	1.5×10^{-5}	0.13 ± 0.01	0.09 ± 0.001
1/16	1	0.75×10^{-5}	0.20 ± 0.02	0.16 ± 0.016
1/16	1/2	3.0×10^{-5}	0.15 ± 0.01	0.18 ± 0.03
1/8	1	1.5×10^{-5}	0.25 ± 0.03	0.19 ± 0.04
1/8	1/2	6.0×10^{-5}	0.20 ± 0.02	0.24 ± 0.05
1/4	1	3.0×10^{-5}	0.35 ± 0.06	0.23 ± 0.05
1/4	1/2	12.0×10^{-5}	0.24 ± 0.03	0.31 ± 0.08

A = Noradrenaline Stimulation (0.25 µg/ml) N = 10.
B = Electrical Stimulation (12 Volts-5 Sec.) N = 8.

TABLE 4.

Effect of Low Sodium Concentrations on Responses of VSM to Noradrenaline.

Dose: (μg/ml)	Response in Modified Krebs Solution (Gm. T.)		P
	Normal $[Na^+]$	Half Normal $[Na^+]$	
0.05	0.05 ± 0.02	0.18 ± 0.04	< 0.01
0.10	0.15 ± 0.04	0.24 ± 0.04	< 0.01
0.50	0.66 ± 0.12	0.92 ± 0.14	< 0.01
1.0	1.10 ± 0.19	1.30 ± 0.20	> 0.05

N = 10.

Normal Sodium = 143 mM.

Half Normal Sodium = 71.5 mM.

P Values indicate the probability that responses of tissues exposed to normal sodium are the same as those exposed to reduced sodium.

TABLE 5.

Tests for Sodium-Calcium Competition Using Potassium as Stimulating Agent.

Fraction of Normal [Ca] & [Na]		Ratio $[Ca^{2+}]:[Na^{+}]^2$	Response (Gm. T.) after exposure to reduced Na for 18 Minutes	Response (Gm. T.) after exposure to reduced Na for 1 Minute
1/24	1	0.5×10^{-5}	0.34 ± 0.04	0.24 ± 0.05
1/24	1/2	2.0×10^{-5}	0.49 ± 0.09	0.30 ± 0.05
1/12	1	1.125×10^{-5}	0.44 ± 0.05	0.41 ± 0.06
1/12	1/2	4.5×10^{-5}	0.70 ± 0.14	0.40 ± 0.06
1/8	1	1.5×10^{-5}	0.74 ± 0.14	0.48 ± 0.07
1/8	1/2	6.0×10^{-5}	0.94 ± 0.20	0.49 ± 0.07
1/6	1	2.125×10^{-5}	0.79 ± 0.16	0.48 ± 0.07
1/6	1/2	8.5×10^{-5}	1.06 ± 0.22	0.64 ± 0.09
1/4	1	3×10^{-5}	1.03 ± 0.21	0.63 ± 0.10
1/4	1/2	12×10^{-5}	1.18 ± 0.24	0.68 ± 0.11
1	1	12.5×10^{-5}	1.44 ± 0.27	0.98 ± 0.18

Stimulus: Potassium Sulfate (54 mM). N = 4

TABLE 6.

Effect of Anions on Response to Potassium Sulfate.

$[K^+]$	8 mM			16 mM			32 mM			64 mM		
Solution	A	B	C	A	B	C	A	B	C	A	B	C
Br												
Gm. T.	0	0	0.2	1.33	1.66	2.15	2.97	3.13	3.20	3.54	3.78	3.75
S.E.±	0	0	0.05	0.23	0.26	0.32	0.38	0.38	0.41	0.35	0.30	0.33
N	8	8	8	8	8	8	8	8	8	4	4	4
p<			0.01		0.01	0.01		0.01	0.02		0.01	0.02
NO_3												
Gm. T.	0	0.28	1.63	1.35	3.02	3.01	2.55	3.02	3.01	2.5	2.87	2.58
S.E.±	0	0.07	0.19	0.21	0.32	0.32	0.28	0.32	0.32	0.44	0.48	0.37
N	8	8	8	8	8	8	8	8	8		0.01	>1.0
p<		0.01	0.01		0.01	0.01		0.01	0.01			
I												
Gm. T.	0	0.78	1.11	0.69	1.90	2.18	1.68	2.27	1.90	2.15	2.60	2.42
S.E.±	0	0.17	0.19	0.12	0.18	0.20	0.23	0.28	0.22	0.11	0.37	0.34
N	8	8	8	8	8	8	8	8	8	4	4	4
p<		0.02	0.01		0.01	0.01		0.01	0.02		0.01	0.01
SCN												
Gm. T.	0	0.25	1.36	0.33	1.17	1.70	0.98	1.53	1.36	0.81	0.90	0.85
S.E.±	0	0.06	0.23	0.02	0.18	0.18	0.09	0.23	0.17	0.22	0.26	0.15
N	8	8	8	8	8	8	8	8	8	4	4	4
p<		0.01	0.01		0.01	0.01		0.01	0.01		0.3	0.7

A = Response in Normal Krebs Solution (Control).
B = Response in 'Anion Solution' after 1 Min. Exposure.
C = Response in 'Anion Solution' after 15 Min. Exposure.

The p values indicate the probability that responses of the tissues exposed to a foreign anion are the same as those of the controls.

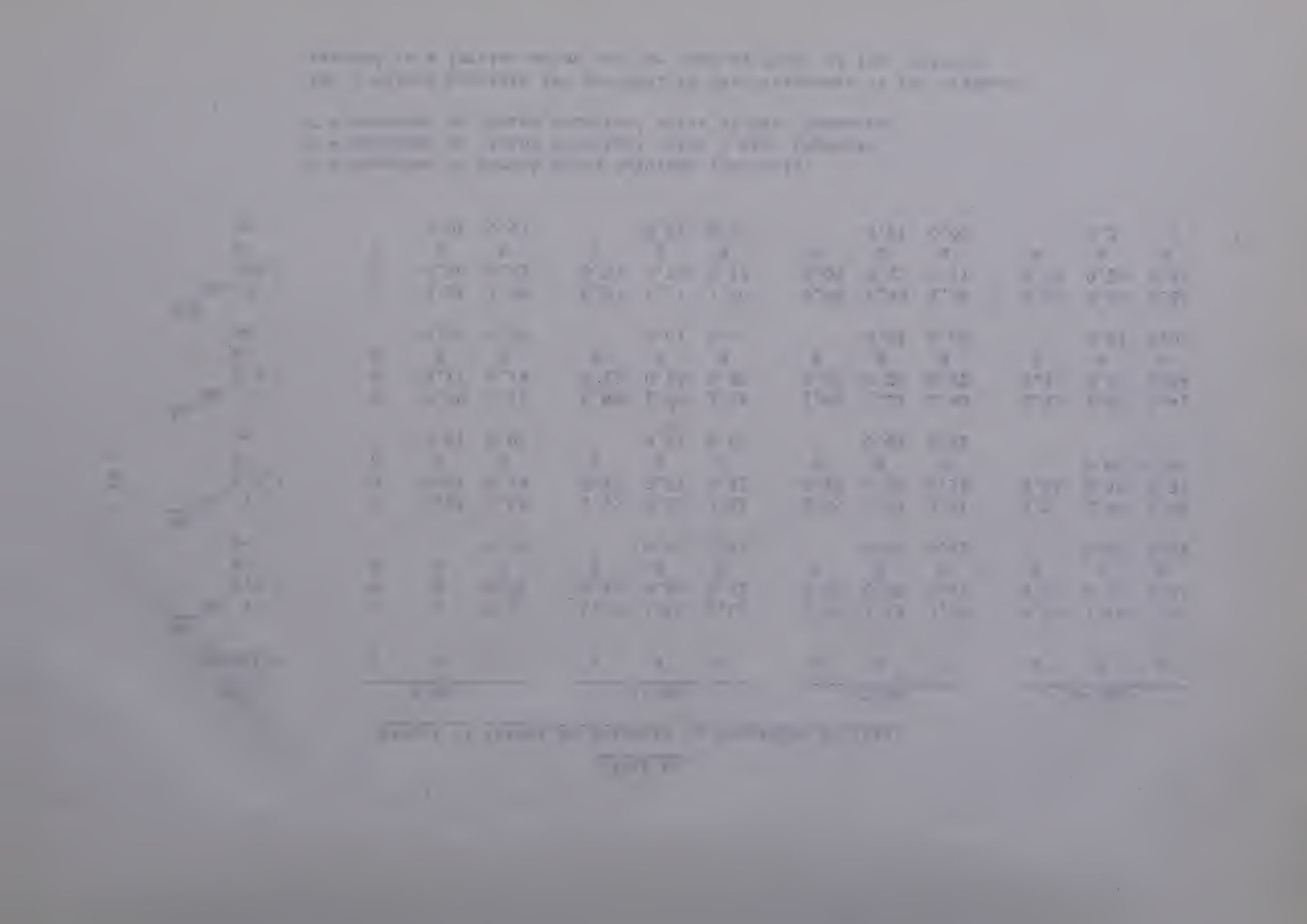

TABLE 7.

Effect of Anions on Response to Noradrenaline.

Dose. NA Solution	0.1 µg/ml.			0.20 µg/ml.			0.40 µg/ml.			1.0 µg/ml.		
	A	B	C	A	B	C	A	B	C	A	B	C
Br												
Gm. T.	2.27	1.92	2.7	3.12	2.68	3.41	3.51	2.97	4.20	4.28	4.08	4.65
S.E.±	0.30	0.31	0.38	0.4	0.42	0.41	0.40	0.38	0.47	0.26	0.27	0.22
N	8	8	8	8	8	8	8	8	8	4	4	4
p<		0.2	0.05		0.01	0.01		0.01	0.02		0.1	0.01
NO_3												
Gm. T.	1.78	2.01	3.11	3.3	3.22	3.98	3.39	3.35	4.15	3.25	2.85	3.90
S.E.±	0.13	0.12	0.15	0.13	0.32	0.07	0.15	0.28	0.18	0.32	0.27	0.28
N	8	8	8	8	8	8	8	8	8	4	4	4
p<		0.4	0.01		0.2	0.01		0.2	0.01		0.02	0.01
I												
Gm. T.	1.66	0.73	0.71	2.19	1.08	1.17	2.29	1.23	1.45	1.71	0.98	1.46
S.E.±	0.18	0.07	0.17	0.15	0.07	0.16	0.21	0.08	0.09	0.11	0.09	0.10
N	8	8	8	8	8	8	8	8	8	4	4	4
p<		0.01	0.05		0.01	0.01		0.01	0.02		0.02	0.01
SCN												
Gm. T.	1.95	0.35	1.38	2.14	0.76	2.07	2.37	1.03	2.47	1.86	0.78	2.11
S.E.±	0.25	0.05	0.18	0.18	0.12	0.20	0.24	0.16	0.25	0.15	0.10	0.20
N	8	8	8	8	8	8	8	8	8	4	4	4
p<		0.01	0.01		0.01	0.9		0.01	0.4		0.01	0.10

A = Response in Normal Krebs Solution (Control).
B = Response in 'Anion Solution' after 1 Min. Exposure.
C = Response in 'Anion Solution' after 15 Min. Exposure.

The p values indicate the probability that responses of the tissues exposed to foreign anion are the same as those of the controls.

TABLE 8.

Effect of Anions on Response to Electrical Stimulation.

Voltage	8 V-5 Sec.			12 V-5 Sec.		
Solution	A	B	C	A	B	C
Br						
Gm. T.	1.30	1.06	1.01	1.86	1.78	1.83
S.E.±	0.20	0.24	0.17	0.14	0.12	0.14
N	4	4	4	4	4	4
NO_3						
Gm. T.	1.30	1.02	1.05	1.43	1.0	0.95
S.E.±	0.20	0.15	0.2	0.23	0.17	0.16
N	4	4	4	4	4	4
I						
Gm. T.	0.68	0.68	0.38	0.24	0.23	0.21
S.E.±	0.23	0.19	0.12	0.07	0.08	0.03
N	4	4	4	4	4	4
SCN						
Gm. T.	0.68	0.31	0.35	0.31	0.26	0.26
S.E.±	0.17	0.09	0.07	0.09	0.08	0.08
N	4	4	4	4	4	4

A = Response in Normal Krebs Solution (Control).
B = Response in 'Anion Solution' after 1 Min. Exposure.
C = Response in 'Anion Solution' after 15 Min. Exposure.

TABLE 9.

Effect of Sulfate Ion on the Response to Noradrenaline, Potassium Sulfate, and Electrical Stimulus.

NA	0.05 µg/ml.			0.10 µg/ml.			0.20 µg/ml.			0.40 µg/ml.		
Solution	A	B	C	A	B	C	A	B	C	A	B	C
Gm. T.	1.74	1.89	1.69	2.68	2.39	2.19	3.4	2.76	2.5	3.42	3.03	2.88
S.E.±	0.08	0.03	0.04	0.11	0.06	0.05	0.12	0.08	0.12	0.08	0.08	0.06
N	4	4	4	4	4	4	4	4	4	4	4	4
p		0.3	0.7		0.1	0.01		0.01	0.01		0.01	0.01

K_2SO_4	12 mM			16 mM			18 mM			32 mM		
Solution	A	B	C	A	B	C	A	B	C	A	B	C
Gm. T.	0.55	0	0	1.55	0.46	0.24	1.96	0.94	0.69	2.10	1.03	0.86
S.E.±	0.11	0	0	0.03	0.04	0.08	0.36	0.07	0.16	0.36	0.09	0.18
N	4	4	4	4	4	4	4	4	4	4	4	4
p<		0.02	0.02		0.02	0.02		0.02	0.04		0.01	0.01

Voltage	8 V-5 Sec.			12 V-5 Sec.		
Solution	A	B	C	A	B	C
Gm. T.	1.65	1.38	0.99	1.84	1.46	0.93
S.E.±	0.15	0.09	0.13	0.16	0.13	0.09
N	4	4	4	4	4	4

A = Response in Normal Krebs Solution (Control).
B = Response in 'Sulfate Solution' after 1 Min. Exposure.
C = Response in 'Sulfate Solution' after 15 Min. Exposure.

The p values indicate the probability that responses of the tissues exposed to foreign anion are the same as those of the controls.